

**HYDROGEN EVOLUTION FROM FIELD PEA BIOLOGICAL NITROGEN FIXATION
AND THE EFFECT ON NITROUS OXIDE PRODUCTION IN SOIL**

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ABSTRACT

Rhizobia in symbiosis with legumes are capable of fixing atmospheric dinitrogen (N_2) into plant available N through biological N fixation (BNF), during which H_2 is produced as an obligate byproduct. Some rhizobia-legume symbioses possess an uptake hydrogenase (HUP) enzyme that is capable of recycling H_2 produced during BNF; this type of symbiosis is referred to as HUP^+ . However, many symbionts do not possess the HUP enzyme (HUP^-) and are therefore unable to recycle H_2 . Consequently, the H_2 diffuses into the soil surrounding the nodules where it can be consumed by H_2 -oxidizing bacteria. There is evidence to suggest that microbial consumption of H_2 in the soil causes increased CO_2 fixation and O_2 consumption in the rhizosphere soil, which could lead to the development of anoxic or hypoxic zones in the soil. These conditions favour denitrification, a process that produces N_2O . The H_2 from HUP^- nodules also has been associated with enhanced plant growth, which may be a non-N benefit associated with planting legumes in rotation with other crops.

Two studies were conducted to look at the effect of H_2 from BNF in field pea (*Pisum sativum* L.) on N_2O production and plant growth enhancement. The first was a growth chamber study where pea plants were grown in the absence of soil. The objectives of the first study were to (i) determine if actively fixing HUP^- pea nodules produced more H_2 than HUP^+ nodules and (ii) if a H_2 -enriched atmosphere around pea nodules stimulated N_2O production. Indeed, actively fixing pea nodules inoculated with HUP^- rhizobia produced significantly more H_2 than nodules inoculated with HUP^+ rhizobia; however, pea nodules inoculated with HUP^- rhizobia and exposed to an enriched H_2 atmosphere were not associated with elevated N_2O .

The second study was completed as a greenhouse experiment where pea plants were grown in soil and N_2O concentrations were monitored over the course of a growing season. The objectives of the greenhouse study were to (i) determine if field pea inoculated with HUP^- rhizobia produced more N_2O than field pea inoculated with HUP^+ rhizobia, and (ii) determine if enhanced plant growth was associated with pea inoculated with HUP^- rhizobia. Rhizosphere N_2O concentrations and surface N_2O flux were measured over the course of a growing season. Pea inoculated with HUP^- rhizobia were not associated with increased N_2O production. As well, there was no enhanced plant growth observed in pea plants inoculated with HUP^- rhizobia compared to HUP^+ rhizobia. This study demonstrated that in the Saskatchewan soil used in this

study, field pea inoculated with HUP⁻ rhizobia did not appear to stimulate N₂O production or enhance plant growth, which further supports the notion that N₂O production associated with legume production is not directly related to BNF.

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DEDICATION

*Dedicated to my beloved companion, Sam,
who is always with me in spirit.*

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agricultural Food Canada
ANOVA	analysis of variance
BNF	biological nitrogen fixation
D	depth
EAC	electron allocation coefficient
ECD	electron capture detector
GC	gas chromatograph
GHG	greenhouse gas
GWP	global warming potential
HE _x R	hydrogen exposure rate
HUP	uptake hydrogenase
L	length
LSD	least significant difference
NF	nitrogen fixation
OD	optical density
PGPR	plant growth-promoting rhizobacteria
SL	season length
TCD	thermal conductivity detector
UHP	ultra high purity
W	width
WFPS	water-filled pore space
YMA	yeast mannitol agar
YMB	yeast mannitol broth

1. INTRODUCTION

The Earth's atmosphere is composed of naturally occurring greenhouse gases (GHGs) that make the planet habitable by trapping solar radiation. However, human activities are increasing atmospheric GHG concentrations, which in turn increase radiative forcing and contribute to global climate change. Increasing atmospheric temperatures are connected to a number of issues such as thawing permafrost, rising sea levels, increasing frequency of intense weather events, and changing vegetation (IPCC, 2007). The three GHGs of greatest concern are carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4). The atmospheric concentrations of all three of these persistent GHGs are increasing because of human activities and will likely continue to increase if changes to global policy are not implemented (IPCC, 2007).

Approximately half of the land surface on Earth is used for agriculture in the form of livestock and crop production (Desjardins et al., 2010). With an ever-increasing global population, the demand for food continues to rise, putting pressure on the agriculture industry to produce more food on a fixed amount of land. Agriculture is responsible for GHG production, primarily CH_4 and N_2O , through crop and animal production (Smith et al., 2007). However, it is difficult to minimize GHGs when the demand for higher yields continues to increase. Inorganic fertilizer and manure are the largest sources of N_2O from agriculture (Ellert and Janzen, 2008; Helgason et al., 2005); however, these are not the only agricultural sources and it is necessary to understand where and how N_2O is produced from other sources.

Legumes are a high protein food used in many developing countries as a dietary staple. Pulse crops, such as lentil and pea, are grown globally to provide an efficient food source and in Canada are grown mainly in Saskatchewan (Hnatowich, 2000). The ability of legumes to fix atmospheric N into plant available N results in a lower demand for inorganic N fertilizer and makes legumes an integral part of the global N cycle. Moreover, interest in quantifying potential N_2O emissions from legumes stems from their N_2 -fixing ability. Biological N fixation (BNF) occurs through a symbiotic relationship between *Rhizobium* bacteria and legume roots (Somasegaran and Hoben, 1994). High N_2O emissions have been measured from legumes (Kilian and Werner, 1996); however, the source of the N_2O emissions remains a point of debate. Whereas most research points to decomposing N-rich residues as the source of these N_2O emissions, recent studies have begun to examine whether N_2O production is directly related to

BNF (Rochette and Janzen, 2005; Zhong et al., 2009).

Hydrogen (H_2) is produced through BNF during the nitrogenase reaction. When legume-rhizobia symbiosis possess the uptake hydrogenase (HUP) enzyme, legume nodules can recycle the H_2 (HUP^+); conversely H_2 will diffuse into the rhizosphere soil when the enzyme is not present (HUP^-) (Evans et al., 1987). When H_2 diffuses into the soil it can increase microbial biomass (Stein et al., 2005), alter microbial community structure (Zhang et al., 2009), improve plant growth of subsequent crops (Dean et al., 2006), enhance CO_2 fixation, and increase O_2 consumption within the rhizosphere (Dong and Layzell, 2001). Enhancing CO_2 fixation and O_2 consumption are thought to create conditions in the soil that may favour denitrification, which could link N_2O emissions to BNF in legumes.

To date, most of the work involving H_2 evolution from HUP^- nodules has focused on soils artificially treated with H_2 , therefore HUP^+ nodules have not been studied in this regard because the HUP enzyme recycles the H_2 evolved. The amount of H_2 evolved from a HUP^- soybean has been calculated and used to treat soil in the absence of growing plants (Dong et al., 2003; Maimaiti et al., 2007). By removing plants, large amounts of soil can be treated with H_2 and then studied under various conditions. However, plant roots in the soil create the rhizosphere, which interacts with the H_2 . For this study, it was important to include the rhizosphere when determining the effect of H_2 from BNF on N_2O emissions. Most work looking at H_2 from BNF has focused on soybean; however, the climate in Saskatchewan is not suited to soybean production. Field pea was chosen to use in this study because it is grown as a successful specialty crop in Saskatchewan.

The conditions that favour N_2O production all occur in the rhizosphere, so it is crucial to measure N_2O in the rhizosphere as well as the soil surface. The hypothesis was that H_2 gas, produced as a byproduct of BNF in field pea, diffuses into the soil and increases N_2O production and emission from the soil. The objective of this study was to determine if H_2 from HUP^- rhizobia and BNF created conditions that resulted in N_2O production in the rhizosphere. An additional objective was to look at plant growth parameters to see if H_2 in the rhizosphere stimulated pea growth. Two HUP^+ rhizobial strains, two HUP^- rhizobial strains, and two controls (a non-nodulating rhizobia and sterilized water) were compared in a series of experiments to meet the objectives.

This thesis is made up of five chapters and four appendices. Following the introduction

(Chapter 1), is a review of the literature (Chapter 2) focusing on GHGs from agriculture, BNF in legumes and H_2 from HUP^- rhizobia. Chapter 3 summarizes two experiments that investigated the effect of H_2 from HUP^- rhizobia on N_2O production. Chapter 4 summarizes the findings, discusses the implications of the results, and suggests future research. A comprehensive list of the literature cited throughout the thesis is presented in Chapter 5.

Because very little work has been done in this area, several foundational experiments had to be completed to provide a starting point. Much of this initial work has been included in the appendices. Appendices A, B and C discuss preliminary experiments that provided important information for the study described in Chapter 3.

2. LITERATURE REVIEW

2.1 Impact of greenhouse gases on the environment

Greenhouse gases (GHGs) in the atmosphere trap radiant heat and allow global temperatures to remain at levels that can support life on Earth. Although GHGs are necessary to create a habitable planet, increasing atmospheric concentrations caused by anthropogenic activities are intensifying the natural process and creating climatic changes (Desjardins et al., 2010). Greenhouse gas emissions are on the rise and are expected to continue increasing if changes in global policy are not implemented. Persistent GHG atmospheric concentrations have been rising since industrialization in developed countries, and include gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) as well as synthetic halocarbons (IPCC, 2007; Rogner et al., 2007). Indeed, CO_2 in the atmosphere has increased by 35% since the advent of large-scale industrialization, largely due to fossil fuel combustion and deforestation, and is the dominant GHG contributing to the increase in atmospheric radiative forcing (IPCC, 2007). Substantial increases caused by anthropogenic activities also have been observed in CH_4 and N_2O . Increases in N_2O are largely attributed to increased N fertilizer use; at the same time, other industrial sources of N_2O have actually decreased since 1970. Land-use change and forestry also contribute to increased GHG concentrations (Rogner et al., 2007).

The persistent GHGs largely responsible for rising global temperatures include CO_2 , CH_4 and N_2O . Each GHG has different radiative forcing capabilities and atmospheric lifetimes; therefore, the global warming potential (GWP) for individual GHGs can be utilized to make comparisons among the various GHGs (IPCC, 2007). All GWP values are for a specific time span and are relative to CO_2 , which has a value of 1. Thus, although CH_4 and N_2O have lower atmospheric concentrations compared to CO_2 , the GWPs of these two GHGs are much greater (IPCC, 2007). Indeed, the GWP (over a 100-year period) for CH_4 is 25 and for N_2O is 298.

As GHG concentrations continue to increase in the atmosphere, the environmental consequences are likely to build. For example, increasing GHG concentrations will continue to amplify the natural greenhouse effect causing an increase in global temperatures, which in turn will alter the frequency and intensity of weather phenomena such as heat waves and droughts (IPCC, 2007). Other impacts also being observed include shrinking ice sheets, thawing permafrost, and changes in the distribution and type of vegetation (IPCC, 2007). As well, N_2O

contributes to stratospheric ozone destruction (Conrad, 1996). These changes, in turn, affect large-scale climatic circulations in the atmosphere and oceans creating a series of feedbacks.

2.1.1 Greenhouse gases from agricultural activities

Agriculture occupies large amounts of land with approximately 40-50% of the global land surface dedicated to agricultural activities. Agricultural soils can be either a source or sink for GHGs depending on the land, activity and GHG of interest (Desjardins et al., 2010). The main GHGs produced by agriculture are N_2O and CH_4 , and agricultural activities such as animal and crop production are responsible for approximately 70% of global N_2O emissions (Mosier, 2001; Smith et al., 2007). In Canada, agricultural soils are the source of about half the N_2O emissions (Helgason et al., 2005). Moreover, because N_2O and CH_4 are produced through different processes, agricultural sources of these GHGs depend on the gas of interest. Most atmospheric gases go through cycles that involve interaction with the biosphere, and soils are particularly important in many of these cycles because they provide habitat and substrate for microorganisms involved in the cycling of N_2O , CH_4 and CO_2 (Conrad, 1996).

Greenhouse gases can originate from a variety of agricultural sources. Sources for agricultural N_2O include inorganic N fertilizers, crop residue decomposition, and livestock manure (Ellert and Janzen, 2008; Helgason et al., 2005). Methane is produced by methanogenic archaea, and, rice paddies represent one of the largest agricultural sources of CH_4 (Mosier et al., 1998; Philippot et al., 2009). However, in Canada, agricultural sources of CH_4 are largely produced from enteric fermentation in ruminant livestock digestion and anaerobic decomposition from manure storage (Desjardins et al., 2010; Mosier et al., 1998).

Management practices can impact the GHG flux associated with agricultural soils (Liebig et al., 2005). In Canada, improved management practices such as reduced tillage, improved nutrient use by crops and animals, and better waste storage solutions have all reduced net GHG emissions from agriculture (Gregorich et al., 2005; Liebig et al., 2005). However, an increasing global human population is creating a greater demand for food, resulting in more livestock, increased N fertilizer use, and more agricultural land conversion and, in turn, increasing total CH_4 and N_2O emissions (Smith et al., 2007).

2.1.2 Nitrous oxide emissions from soil

Nitrous oxide emitted from soil is the predominant GHG from agriculture and the majority of N_2O is associated with N fertilizer use and manure application (Mosier, 2001). Whereas environmental factors determine whether a soil is an overall source or sink for GHGs, soils are generally considered to be net sources for N_2O (Conrad, 1996). Nitrous oxide can be produced through nitrification or denitrification processes, which means that both nitrifying and denitrifying prokaryotes are of interest when quantifying N_2O from soil (Conrad, 1996; Philippot et al., 2009).

Nitrification is a two-step aerobic process typically carried out by chemolithotrophic bacteria responsible for converting ammonia (NH_3) to nitrite (NO_2^-) followed by converting NO_2^- to nitrate (NO_3^-) (Philippot et al., 2009; Wrage et al., 2001). Primary nitrifiers that oxidize NH_3 carry out the first step, while the second step is carried out by secondary nitrifiers. Both groups of bacteria are grouped together as Nitrobacteriaceae. During nitrification, intermediates such as hydroxylamine (NH_2OH) and NO_2^- are produced. These intermediates can undergo chemical decomposition in a process referred to as chemodenitrification and produce N_2O (Wrage et al., 2001). Fungi often carry out heterotrophic nitrification, but the process has the same intermediates and products as autotrophic nitrification (Wrage et al., 2001).

Denitrification is a stepwise process capable of returning N_2 to the atmosphere by reducing NO_3^- to NO_2^- then to nitric oxide (NO) followed by N_2O and N_2 (Tiedje, 1988). Denitrifiers are predominantly heterotrophic microorganisms that are facultative anaerobes able to deal with low-oxygen conditions (Tiedje, 1988; Wrage et al., 2001). Nitrous oxide is an intermediate product of denitrification, which can be produced in soil when conditions, such as low oxygen, sufficient NO_3^- and available organic C, favour its production (O'Hara and Daniel, 1985; Philippot et al., 2009; Wrage et al., 2001).

Nitrifier denitrification is also a potential pathway for N_2O from agricultural systems (Wrage et al., 2001). In nitrifier denitrification, the two-step nitrification process is altered; the oxidation of NH_3 to NO_2^- proceeds, but is then followed by the reduction of NO_2^- to N_2O and N_2 . Both of these steps are carried out by autotrophic NH_3 -oxidizers (Wrage et al., 2001). Only primary nitrifiers carry out nitrifier denitrification and no NO_3^- is produced during the process. Nitrifier denitrification appears to occur in low-oxygen conditions where autotrophic nitrification is limited (Wrage et al., 2001).

The primary factors influencing N₂O production in the rhizosphere are N application, oxygen partial pressure, and carbon availability (Philippot et al., 2009; Tiedje, 1988). The pH can also affect N₂O production from denitrification with more N₂O produced at low pH (Philippot et al., 2009; Wrage et al., 2001). However, plant roots, plant species and soil type also affect N₂O production (Philippot et al., 2009). Inefficient N fertilizer use can cause N₂O emissions from agricultural soils and will result in a burst of N₂O following N fertilizer application (Ellert and Janzen, 2008).

Where N₂O is produced in the rhizosphere is still unclear; however, the processes responsible for N₂O may determine what factors have spatial influence. Denitrification is driven by root-derived organic compounds and low oxygen availability, whereas, nitrification is influenced by competition for NH₄⁺. Temporal, as well as, spatial changes may determine which process is responsible for N₂O production (Philippot et al., 2009). Interactions and drivers at the rhizosphere level are likely to differ from those at the field scale. Gas fluxes are challenging to study due to the high degree of temporal and spatial variability, as well, soils are often viewed on a macro-scale, but many of the processes occur at microscopic levels (Conrad, 1996).

2.2 Importance of pulse crops in Saskatchewan

Pea (*Pisum sativum* L.), bean (*Phaseolus vulgaris* L.), lentil (*Lens culinaris* L.), chickpea (*Cicer arietinum* L.), and faba bean (*Vicia faba* L.) are examples of edible legume seeds commonly referred to as pulse crops (Somasegaran and Hoben, 1994). Seeds from pulses have been a dietary staple around the world for thousands of years and are still an important protein source (Ghosh et al., 2002). The three pulses that dominate growing area and production worldwide are bean, pea and chickpea. In Canada, however, pea, lentil, and chickpea are the major pulse crops. Canada is also the global leader in exportation of these three pulses with the majority of these crops grown in Saskatchewan (Hnatowich, 2000). This reflects the fact that cold winters and dry summers common in Saskatchewan are well suited to pulse production (Slinkard and Drew, 1988). Pulse crops, when grown in rotation with other crops, offer advantages such as increasing available soil N, adding N-rich residues to the soil, and breaking cereal crop disease cycles (Stevenson and van Kessel, 1996). Crops (e.g., cereals, flax or canola) grown subsequent to a pulse crop in rotation typically have higher yields and require less N fertilizer compared to crop rotations that do not include pulses (Slinkard and Drew, 1988;

Stevenson and van Kessel, 1996). The reduced N requirement for subsequent crops does not fully explain the benefit of pulse crops in rotation and is attributed to increased microbial activity, reduced weed pressure, and nutrient availability (Stevenson and van Kessel, 1996; Zhang et al., 2009).

2.3 Biological nitrogen fixation

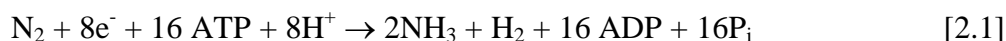
Legumes, including pulse crops, form symbiotic relationships with *Rhizobium* bacteria to fix atmospheric N₂ into plant available forms with both the rhizobia and the plant benefitting from the association. Rhizobia are free-living, Gram-negative soil bacteria that, with the exception of a few strains, are typically not capable of fixing N unless in association with legume roots (Somasegaran and Hoben, 1994; Vincent, 1970). Symbiosis occurs when rhizobia infect legume roots by entering root hairs and forming nodules at the site of infection. Nodulation is governed by chemical signals between plant roots and rhizobial bacteria. Root hairs are infected by rhizobia, which induce the root to form an infection thread and allow the bacteria to enter the root hair. Eventually, rhizobia enter into cortical cells within the root and become bacteroids. Cortical cells form nodules, which contain both infected and uninfected cells (Gage, 2009). Active N₂-fixing nodules have pink or red interiors caused by iron in the protein leghemoglobin (Somasegaran and Hoben, 1994). Bacteroids within nodules convert N₂ into plant available forms of N using carbohydrates derived from the host plant. Legumes require reduced N fertilizer inputs compared to other crops resulting in economic and environmental benefits (Hnatowich, 2000).

The rhizobia-legume association is species-specific meaning that a particular rhizobial species can effectively nodulate only certain legume species. Sufficient numbers of rhizobia are required in the soil to infect and nodulate legumes in order to fix substantial amounts of N (Vincent, 1970). Legume crops are typically inoculated with an appropriate strain of rhizobia to ensure presence and quantity for effective biological nitrogen fixation (BNF). The *Rhizobium* species specific to pea, lentil and faba bean is *Rhizobium leguminosarum* biovar *viciae* whereas the *Rhizobium* species specific to soybean is *Bradyrhizobium japonicum* (Somasegaran and Hoben, 1994). However, even within a certain species, *Rhizobium* strains can be suited to different environments or different crops, as is the case with *R. leguminosarum*.

Commercial inoculants can be formulated from a single *Rhizobium* strain or multiple strains

depending on its intended use. Multiple *R. leguminosarum* strains are often combined into a single inoculant to be applied to pea and/or lentil seeds (Hnatowich, 2000). Commercial inoculants are applied as peat-, liquid-, or granular-based formulations directly to the seed or soil. Much of the BNF research has focused on understanding and improving rhizobial strains to develop better inoculants for legume crops capable of competing with indigenous soil rhizobia (Golding and Dong, 2010).

Biological N fixation in legume-rhizobia symbiosis occurs through the nitrogenase reaction, which is a complex of two enzymes responsible for reducing N₂ to ammonia (NH₃). Ammonium (NH₄⁺) is formed by the protonation of NH₃ and is a plant available form of N used by the plant during the conversion of metabolites into amino acids which are then synthesized into proteins (Somasegaran and Hoben, 1994). The enzymes involved in this reaction contain an iron protein and an iron-molybdenum protein that are synthesized in the cytosol of bacteroids in the nodule (Somasegaran and Hoben, 1994) producing H₂ as an obligate byproduct (Equation 2.1) (Dong and Layzell, 2001; Strodtman and Emerich, 2009). This process is energy intensive for the host plant and based on several estimates accounts for approximately 5% of net photosynthesis (Dong and Layzell, 2001).



2.3.1 Quantifying nitrous oxide emissions from pulse crops

The relationship between N₂O emissions and BNF in pulse crops is not well understood; however, both are important N transformations and there have been a number of studies to quantify N₂O emissions from pulse crops. Several pathways have been suggested for how BNF may be linked to N₂O emissions; these include nitrification using biologically fixed N (Galloway, 1998), N-rich residue decomposition (Ellert and Janzen, 2008), or directly through rhizobial denitrification (O'Hara and Daniel, 1985). The abundance of N-rich plant material, above- and belowground, makes pulse crops a potential source of N₂O emissions. Moreover, because agriculture is responsible for such a high percentage of N₂O emissions, it is important to identify and inventory any and all sources and sinks. The N₂ fixing ability of pulse crops results in reduced inorganic N fertilizer requirements, which reduces potential N₂O emissions from fertilizer applications. However, there has been considerable interest in N₂O production that may

result from BNF and offset the benefit of reduced fertilizer applications.

Some studies found that actively fixing legumes did not increase N₂O emissions (Ellert and Janzen, 2008; Zhong et al., 2009). When N₂O production from inoculated and non-inoculated pea, lentil and soybean, grown in Leonard jars, was compared, there were no significant differences in N₂O production between the inoculated and non-inoculated treatments (Zhong et al., 2009). In the same study, when actively fixing pulses were compared to a non-fixing cereal (wheat) both grown in a Saskatchewan Chernozemic soil, the pulses did not stimulate N₂O emissions when compared to the wheat treatments. The conclusion of the study was N₂O production may not be directly linked to BNF under the specific conditions used in the study (Zhong et al., 2009). Although some rhizobia are known to denitrify, such as *B. japonicum* isolate G49, the two *R. leguminosarum* isolates (99A1 and RGP2) used in the study were not capable of denitrifying in pure culture and did not produce N₂O (Zhong et al., 2009).

Although there has been considerable interest in quantifying N₂O emissions from BNF in pulses, often the N₂O released from decaying legume residues is more substantial (Ellert and Janzen, 2008). Alfalfa plots emitted more than twofold N₂O emissions over an entire growing season compared to corn plots. This appeared to be strongly influenced by previously fixed N being released to the soil during decomposition, but little N₂O was associated with active BNF (Ellert and Janzen, 2008). Similar results were observed in soybean nodules where very little N₂O was detected from fresh, healthy nodules; however, much higher amounts of N₂O were detected from degraded nodules suggesting that decomposing nodules may be a source of N₂O in the legume rhizosphere (Inaba et al., 2009). Late-growth nodulated soybean roots produced significantly more N₂O than non-nodulated roots (Inaba et al., 2009). However, there was little evidence to show that active legume nodules were able to produce N₂O.

Other studies have found active N₂ fixing legumes were associated with increased denitrification rates and N₂O emissions. For example, soil with nodulated faba bean roots showed four times higher N₂O-N production than soil from non-nodulated faba beans (Kilian and Werner, 1996). As well, the soil from N₂-fixing faba beans had higher rates of denitrification than non-fixing plants (Kilian and Werner, 1996). Another study using soybean, black gram, lentil and Bengal gram found that all four legume crops produced significantly more total N₂O-N over the growing season than the control treatments (Ghosh et al., 2002). The same study found that total N₂O-N emissions from the four legume crops were higher than emissions from rice and

wheat crops grown under the same conditions (Ghosh et al., 2002).

2.4 Uptake hydrogenase in legumes

Uptake hydrogenase (HUP) is an enzyme that is present in some rhizobia, and when present the nodules are referred to as HUP⁺ (Robson and Postgate, 1980). Many rhizobial bacteria lack the HUP enzyme; in this case, the nodules are referred to as HUP⁻ (Robson and Postgate, 1980). When the HUP enzyme is present (Figure 2.1A), most of the H₂ produced through BNF is oxidized and a portion of the energy used during BNF is recovered, although there is no energy recovered in *R. leguminosarum* bacteroids (Evans et al., 1987; Nelson and Salminen, 1982). However, in HUP⁻ nodules (Figure 2.1B), the H₂ produced through BNF cannot be recycled because the enzyme is not present (Evans et al., 1987). Under these conditions, the H₂ diffuses into the soil around the HUP⁻ nodules (Lafavre and Focht, 1983). Most clover and alfalfa, as well as many soybean and pea symbioses are HUP⁻ (Hunt and Layzell, 1993). The HUP activity in legumes does vary; however, many rhizobia are predominantly HUP⁻; e.g., 75% of rhizobia used with soybean are HUP⁻ (Uratsu et al., 1982), and the majority of rhizobia (*Rhizobium leguminosarum*) forming symbioses with pea and lentil are also HUP⁻ (Nelson and Child, 1981). Conversely, cowpea rhizobia appear to be dominated by HUP⁺ strains (Lafavre and Focht, 1983).

Although substantial amounts of H₂ can be produced by HUP⁻ nodules, no H₂ escapes the root-soil system as most of the H₂ is consumed by soil microorganisms within 3 – 4.5 cm of the nodules (Figure 2.1B) (Lafavre and Focht, 1983). For example, H₂ production from HUP⁻ soybean was calculated to range between 215,000 to 240,000 L ha⁻¹ season⁻¹ (Dong et al., 2003; Peoples et al., 2008). Similar calculations have been done using soybean as a model to predict the amount of H₂ evolved from HUP⁻ legume nodules. Based on these calculations an average soil H₂ exposure rate from HUP⁻ legume nodules ranges between 30 to 254 nmol H₂ cm⁻³ soil h⁻¹ for soils within 4 cm of legume nodules (Dong and Layzell, 2001).

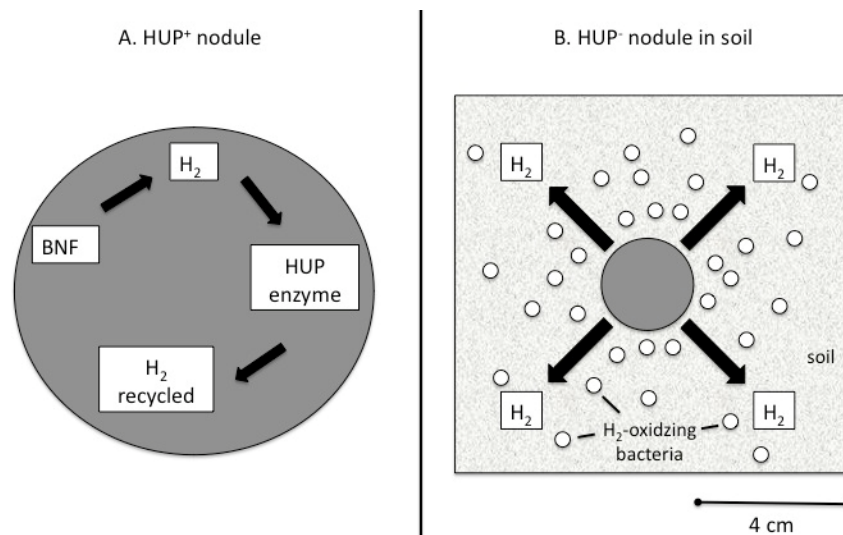


Figure 2.1. The difference between a HUP⁺ and HUP⁻ nodule. In a HUP⁺ nodule (A), the H₂ produced as a byproduct of BNF is captured by the HUP enzyme, which then recycles the H₂ and recovers a portion of the energy used to produce the H₂. In a HUP⁻ nodule (B), the HUP enzyme is not present, so the H₂ produced through BNF diffuses from the nodule into the surrounding soil where it is consumed by H₂-oxidizing bacteria.

2.4.1 Effect of hydrogen gas in the rhizosphere

Hydrogen produced by HUP⁻ nodule symbionts may explain, in part, why HUP⁻ rhizobia have not been selected against through evolutionary processes. Indeed, although HUP⁺ rhizobia may be more energy efficient, HUP⁻ rhizobia may offer other benefits to the plants (Dong et al., 2003). Enhanced plant growth was observed in soybean, wheat, barley and canola when plants were grown in soil that had previously been treated with H₂ at a rate similar to that evolved from HUP⁻ soybean nodules. The majority of the plant growth increases were observed in plant shoots and an increase in tiller number on wheat and barley plants, and were attributed to H₂-oxidizing microorganisms (Dong et al., 2003). Hydrogen-oxidizing microorganisms are stimulated by the presence of H₂ in the soil from HUP⁻ nodules evolving H₂ (Lafavre and Focht, 1983). Thus, soil surrounding HUP⁻ nodules has increased H₂ oxidation capacity, which develops in approximately seven to ten days during exposure to H₂ (Dong and Layzell, 2001; Lafavre and Focht, 1983). This suggests that there are other benefits to growing legumes in rotation with other crops besides the N₂-fixing ability (Hnatowich, 2000). For example, the H₂-oxidizing microorganisms may be able to act as plant growth-promoting rhizobacteria (PGPR) (Dong et al., 2003; Maimaiti et al., 2007).

Microorganisms in the soil develop H₂ uptake capabilities within several days of exposure to

H₂ either in the form of artificial H₂ treatment or with HUP⁻ status nodules present (Dong et al., 2003). Hydrogen in the rhizosphere can influence rhizobial biomass and increase rates of O₂ consumption and CO₂ fixation (Dong and Layzell, 2001; Zhang et al., 2009). Many studies have focused on artificial H₂ soil treatment using compressed air; however, similar trends in increased plant growth were observed in barley grown in rotation with HUP⁻ soybeans. Barley plants grown subsequent to soybeans inoculated with HUP⁻ *B. japonicum* had significantly higher yields than barley following soybeans inoculated with HUP⁺ rhizobia (Dean et al., 2006). Natural H₂ evolution from HUP⁻ soybean nodules had an impact on plant growth of the succeeding crops (Dean et al., 2006). Soil microbial communities around HUP⁻ soybean nodules developed much greater H₂ uptake abilities than those around HUP⁺ and non-inoculated soybeans which indicated high numbers of H₂-oxidizing microbial populations around HUP⁻ nodules compared to HUP⁺ nodules and roots lacking nodules (Dean et al., 2006).

2.4.2 Influence of biological nitrogen fixation and hydrogen on soil microbial activities

Hydrogen can provide a reliable substrate for a number of bacteria (H₂-oxidizing bacteria) in the rhizosphere because it is produced and metabolized within the rhizosphere (Conrad, 1996). Moreover, soils exposed to H₂ demonstrate increased rates of H₂ uptake that can be linked to increased microbial activity and PGPR (Maimaiti et al., 2007; Zhang et al., 2009). Rhizosphere bacterial community structure is altered by H₂ metabolism; e.g., soil adjacent to HUP⁻ soybean nodules exhibited greater H₂ uptake rates than soil adjacent to HUP⁺ nodules (Zhang et al., 2009). When soil from a greenhouse study comparing HUP⁺ and HUP⁻ soybeans was compared to the same soil that had been artificially treated with H₂, the bacterial community structures differed, suggesting that soybean root activity affected rhizosphere bacterial community structure in a way that could not be simulated using H₂ alone (Zhang et al., 2009).

2.4.3 Effect of hydrogen on nitrous oxide production in the rhizosphere

The presence of H₂ in soil around HUP⁻ nodules may provide a link between BNF and N₂O emissions from pulse crops. As H₂ uptake increases in soil, O₂ consumption also increases and CO₂ evolution decreases (Dong and Layzell, 2001). This indicates that CO₂ fixation accompanies H₂ oxidation and that soil CO₂ exchange switches from production to consumption at high H₂ concentrations (Dong and Layzell, 2001). Based on Dong and Layzell's (2001) calculations,

40% of H_2 electrons are allocated to CO_2 reduction and 60% of H_2 electrons go to O_2 reduction. Hydrogen oxidation also is linked to organic carbon increases in rhizosphere soil around legume nodules (Dong and Layzell, 2001). As well, it has been proposed that H_2 oxidation near HUP⁻ nodules could create hypoxic or anoxic zones within the soil that could favour denitrification and increased N_2O production. Indeed, H_2 -treated soil reportedly produced a 10-fold increase in N_2O compared to air-treated soil (Golding and Dong, 2010). To date, research in this area has focused primarily on soybean and bulk soil, as opposed to rhizosphere soil, artificially treated with H_2 , with little work focusing on other legumes (e.g., pea) or nodule-evolved H_2 in rhizosphere soils.

3. THE EFFECT OF *RHIZOBIUM LEGUMINOSARUM* ON HYDROGEN AND NITROUS OXIDE PRODUCTION IN THE RHIZOSPHERE

3.1 Introduction

Pulse crops are an important part of the Saskatchewan agriculture sector with field pea as one of the main specialty crops grown in the province with approximately 629,286 ha seeded in Saskatchewan in 2011 (S.M.A., 2011). One of the benefits of growing pulse crops, such as pea, is the low N fertilizer demand resulting from the ability of legumes to fix atmospheric N_2 into plant available forms through a symbiotic relationship with rhizobial bacteria (Somasegaran and Hoben, 1994). Rhizobia infect legumes by entering the root hairs and forming nodules at the site of infection. Actively N-fixing nodules convert N_2 into plant available NH_4^+ through the nitrogenase reaction (Somasegaran and Hoben, 1994). The relationship between legumes and rhizobia is species-specific, and although rhizobia are free-living bacteria found in soil, legume crops are often inoculated with a compatible species of rhizobia to ensure successful inoculation and nodule formation. *Rhizobium leguminosarum* biovar *viciae* is the rhizobial species compatible with field pea and lentil, however even within a single species there are *Rhizobium* strains that are better suited to various environmental conditions (Somasegaran and Hoben, 1994).

During biological N fixation (BNF), H_2 is produced as an obligate by-product during the nitrogenase reaction. The production of H_2 in legumes can use 5% of the energy obtained from net photosynthesis, which represents an energy intensive process for the host plant (Dong and Layzell, 2001). Individual strains of rhizobia either possess or lack the uptake hydrogenase (HUP) enzyme, which catalyzes the oxidation of H_2 to protons (H^+) and electrons (e^-) where electrons are subsequently used to produce energy via oxidative phosphorylation. Rhizobia with the HUP enzyme are referred to as HUP-positive (HUP^+) and are capable of recouping a portion of the energy used to produce the H_2 . Rhizobia that lack the HUP enzyme are referred to as HUP-negative (HUP^-). If rhizobia are HUP^- , the enzyme is not present and the H_2 produced through the nitrogenase reaction diffuses from the nodule into the surrounding rhizosphere soil (Evans et al., 1987; Lafavre and Focht, 1983). Hydrogen-oxidizing bacteria in the rhizosphere metabolize H_2 within 3 to 4.5 cm of the nodule and the H_2 does not leave the plant-soil system (Lafavre and Focht, 1983).

Hydrogen in the soil surrounding HUP⁻ nodules is associated with increased microbial biomass (Stein et al., 2005), altered microbial community structure (Zhang et al., 2009), stimulated plant growth (Dong et al., 2003) and increased CO₂ fixation and O₂ consumption in the rhizosphere (Dong and Layzell, 2001). Hydrogen diffusing into the soil causes H₂-oxidizing bacteria to undergo rapid multiplication around HUP⁻ legume nodules, as well as creates changes in microbial community structure (Stein et al., 2005; Zhang et al., 2009). Hydrogen from HUP⁻ nodules has been connected with increased plant growth in subsequent non-legume crops (Dean et al., 2006; Dong et al., 2003). In addition, microbial H₂ uptake by H₂-oxidizing bacteria has been associated with increased CO₂ fixation and O₂ consumption in the rhizosphere soil around HUP⁻ nodules (Dong and Layzell, 2001; Stein et al., 2005). Although some H₂-oxidizing bacteria have been isolated (Maimaiti et al., 2007), it is not clear if H₂-oxidizers fix more CO₂ in the presence of H₂ or whether H₂ stimulates an increase in several microbial populations. Increases in CO₂ fixation and O₂ uptake may create hypoxic or anoxic zones in the soil, providing conditions that favour denitrification (Dong and Layzell, 2001; Golding and Dong, 2010).

Nitrous oxide (N₂O) is a potent greenhouse gas, with a 100-year global warming potential 298× greater than that of CO₂ (IPCC, 2007). Agricultural soils are estimated to produce approximately one-half of all N₂O emissions in Canada (Helgason et al., 2005), with the N₂O being produced through microbial transformations (nitrification and/or denitrification) of soil- and fertilizer-N. Although N₂O can be produced from legume crops there is some doubt over whether there is a direct connection between N₂O production and BNF (Rochette and Janzen, 2005). There are several pathways for N₂O production from legumes: nitrification of biologically fixed N (Galloway, 1998), decomposition of N-rich residues (Ellert and Janzen, 2008) or direct denitrification by rhizobia (O'Hara and Daniel, 1985). Moreover, Golding and Dong (2010) recently reported a ten-fold increase in N₂O emissions from soil treated with H₂ at a rate similar to that evolved from soybean. To date, however, this phenomenon has not been reported for other crop/*Rhizobium* combinations. Consequently, how N₂O production in pulse cropping systems is impacted by H₂ produced/released from HUP⁻ nodules remains unclear.

To help elucidate the role of H₂ in N₂O production in field pea, two studies were conducted to (i) determine if actively fixing HUP⁻ pea nodules produce more H₂ than HUP⁺ nodules, (ii) determine if a H₂-enriched atmosphere around pea nodules stimulates N₂O production, (iii) compare the effect of rhizobial HUP status on N₂O emissions from field pea grown in soil, and

(iv) determine if HUP⁻ rhizobia enhance plant growth.

3.2 Materials and Methods

3.2.1 *Rhizobium leguminosarum* strains and growth conditions

Five *R. leguminosarum* biovar *viciae* strains were used throughout these studies. Three isogenic strains, a HUP⁺ (128C53), a HUP⁻ (3855PJB5J1), and a non-nodulating strain (B151), were selected. Strains 3855PJB5J1 (henceforth referred to as PJB5J1) and B151 were developed from the parent strain (128C53) by Dr. M. Hynes at the University of Calgary, AB, Canada. Two additional rhizobial strains 128C52 (HUP⁺) and 128C79 (HUP⁻) also were selected. Strains 128C53, 128C52, and 128C79 were obtained from Dr. J. Germida's collection at the University of Saskatchewan, Canada. Further discussion of the *R. leguminosarum* strains can be found in Appendix A. In addition to the five *R. leguminosarum* strains, sterilized water was used as a control.

Rhizobial cultures were incubated in flasks of sterilized yeast mannitol broth (YMB) at room temperature on a rotary shaker at 100 rpm (Somasegaran and Hoben, 1994). Growth curves for each *R. leguminosarum* strain were determined by plotting optical density (OD) versus time (Appendix A). The OD readings were converted to cell counts [i.e., colony forming units (cfu) mL⁻¹] based on calibration curves prepared for each *R. leguminosarum* strain (Somasegaran and Hoben, 1994). Throughout this study, OD readings were used to monitor the growth of each culture and enable known concentrations of each *Rhizobium* strain to be applied to pea seeds prior to planting. The target cell concentration for each culture was set at approximately 1×10^8 cfu seed⁻¹ for each experiment (Mabrouk et al., 2007).

The presence or absence of the HUP enzyme was confirmed using the methylene blue reduction assay on nodulating *R. leguminosarum* strains (Palacios et al., 1988). Strains 128C52 and 128C53 were confirmed as HUP⁺ and 128C79 and PJB5J1 were confirmed as HUP⁻ (Appendix B).

3.2.2 Ambient and H₂-enriched atmosphere study

3.2.2.1 Leonard jar assemblies

The first study was conducted in a growth chamber and used modified Leonard jar assemblies to grow pea plants without soil (Somasegaran and Hoben, 1994). Leonard jars are composed of a

1.0 L Mason jar and an inverted longneck 341 mL amber bottle with the bottom removed. The neck of the amber bottle is placed in the mouth of the Mason jar. The inverted bottle has a cotton wick installed through the neck of the bottle extending into the Mason jar. The body of the amber bottle is filled with sand, which acts as the rooting substrate; a cotton ball in the neck of the bottle keeps the sand and cotton wick in place. The Mason jar serves as a reservoir for nutrient solution, which is wicked up the cotton wick into the sand. The entire unit is wrapped in aluminum foil and autoclaved for 60 min at 121°C (Somasegaran and Hoben, 1994). The nutrient solution used in the reservoir was Fahraeus N-free nutrient solution (Somasegaran and Hoben, 1994; Vincent, 1970).

Pea (*Pisum sativum* cv. CDC Meadow) seeds were surface sterilized by rinsing with 95% ethanol for 10 s followed by rinsing with 2.5% sodium hypochlorite for 4 min and then six rinses with sterile water. After surface sterilization, seeds were refrigerated and soaked in sterile water for 4 h to allow seeds to imbibe (Somasegaran and Hoben, 1994). Seeds were pre-germinated for two to three days under aseptic conditions in Petri dishes containing filter paper moistened with water. Immediately before planting, sprouted pea seeds were inoculated with one of the *R. leguminosarum* treatments or sterile water by evenly applying 0.5 mL of the *Rhizobium* culture to four sprouted seedlings (Table 3.1). Seedlings were inoculated prior to planting to ensure even application of rhizobia to each seed. After inoculation, one seedling was transplanted into each Leonard jar.

Table 3.1. Estimated *R. leguminosarum* population applied to pea seedlings at planting. Excised roots were exposed to an ambient air atmosphere in the first experiment and to a H₂-enriched atmosphere in the second experiment.

<i>R. leguminosarum</i> strain	HUP status	Rhizobia population at planting cfu seed ⁻¹
	<u>Ambient air atmosphere</u>	
128C52	+	0.92×10^8
128C53	+	1.52×10^8
128C79	-	1.56×10^8
PJB5J1	-	1.39×10^8
B151	n/a	1.08×10^8
	<u>H₂-enriched atmosphere</u>	
128C52	+	0.88×10^8
128C53	+	0.97×10^8
128C79	-	1.52×10^8
PJB5J1	-	1.22×10^8
B151	n/a	1.43×10^8

The study was conducted as two experiments. In both experiments, pea were grown for four weeks in a growth chamber with a day/night temperature of 24°C/21°C and day/night length of 16 h/8 h. Each treatment was replicated four times for each experiment. In the first experiment, roots were excised after four weeks and exposed to an ambient air atmosphere; in the second experiment, roots were excised after four weeks and exposed to a H₂-enriched atmosphere.

3.2.2.2 *Root and nodule sampling*

Pea plants were harvested after four weeks of growth and roots and nodules were washed, blotted dry, and sealed in 250 mL Pyrex media jars (VWR International, LLC, Mississauga, ON) with lids fitted with self-sealing septa. The first experiment used ambient air as the atmosphere in media jars. Headspace samples were collected 30, 60, 90 and 120 min after sealing using a 20 mL syringe and needle; headspace samples were injected into pre-evacuated 12 mL Exetainer vials (Labco Ltd., Lampeter, Ceredigion, UK) resulting in a pressurized vial. After each gas sample was collected, an equal volume of ambient air was injected back into the media jar to replace the volume of air removed. Similar methods have been used to measure N₂O or H₂ evolved from fresh roots and nodules (Inaba et al., 2009; Nelson and Child, 1981).

The second experiment followed the same sampling procedure, but used a H₂-enriched atmosphere in the media jars. The H₂ atmosphere was initially created by injecting 0.75 mL of 5% H₂ in N₂ into the media jars once the roots and nodules were sealed inside. This created a 100 ppmv H₂-enriched atmosphere simulating the effect of high H₂ concentration around HUP⁺ nodules. After each gas sample was drawn from the media jar, an equal volume of H₂-enriched air was injected into the media jars to replace the volume removed.

In addition to the six treatments, empty media jars were included as blanks for each experiment in the study to establish background H₂ and N₂O levels. Under ambient conditions, the blank media jars contained ambient air and were sampled in the same manner as the media jars containing excised roots and nodules. In the H₂-enriched experiment, the blank media jars contained the same H₂-enriched atmosphere as the jars containing the roots and nodules. All gas samples were analyzed for H₂ and N₂O using gas chromatography; parameters for gas sample analysis are described in Section 3.2.4. Roots and nodules were separated, dried at 60°C, and weighed once gas sampling was completed.

3.2.3 Greenhouse study

A greenhouse study using pea inoculated with the five rhizobial strains used in the growth chamber study, or a sterile water control, was conducted to determine the effect of HUP status on N₂O emissions and plant growth parameters. Soil was collected from a long-term, wheat-fallow rotation at the Agriculture and Agricultural Food Canada (AAFC) research station in Swift Current, SK (50°17'N, 107°48'W) on May 3, 2010. The soil is classified as an Orthic Brown Chernozem with a silty loam texture and pH of 7.1 (Table 3.2). Soil was collected from 0 to 15 cm, air-dried, homogenized, sieved to 2 mm and mixed with silica sand in a 1:1 (w/w) ratio.

Table 3.2. Soil characteristics and nutrient levels[†] for soil collected from Swift Current, SK used in greenhouse study. Soil was mixed with silica sand before being used in pots.

Texture	pH	NO ₃ -N	P	K	SO ₄ -S
		kg ha ⁻¹			
silt loam	7.1	21	65	493	8

[†] Soil analyzed by ALS Laboratory Group Agricultural Services (Saskatoon, SK) in 2011.

3.2.3.1 Greenhouse pot design

Acrylic pots with a 12.7 cm i.d. and 30 cm height were built for the greenhouse study. Each acrylic pot had two airtight Swagelok gas sampling bulkhead connectors containing self-sealing septa installed in the walls of the pot, with the bulkheads connected to a 150-cm coil of gas-permeable, platinum-cured Tygon® silicone tubing with a 4.78 mm i.d. (Cole-Parmer Canada Inc., Montreal, QC) (Figure 3.1). The bulkheads created sampling ports used to collect gas samples. The silicone tubing was attached to a support structure that held the tubing in a coil and prevented it from collapsing when soil was added to the pot. The support structure was made of four plastic rods attached to two metal rings and is shown in Figure 3.1. The tubing was located in the area of highest root density within the pot [i.e., the 5- to 20-cm depth interval (Gan et al., 2009)]. Each pot was filled with the soil-sand mixture to a bulk density of 1.54 g cm⁻³. The soil mixture was maintained at 65% water-filled pore space (WFPS) to optimize N₂O production from both nitrification and denitrification (Bateman and Baggs, 2005).

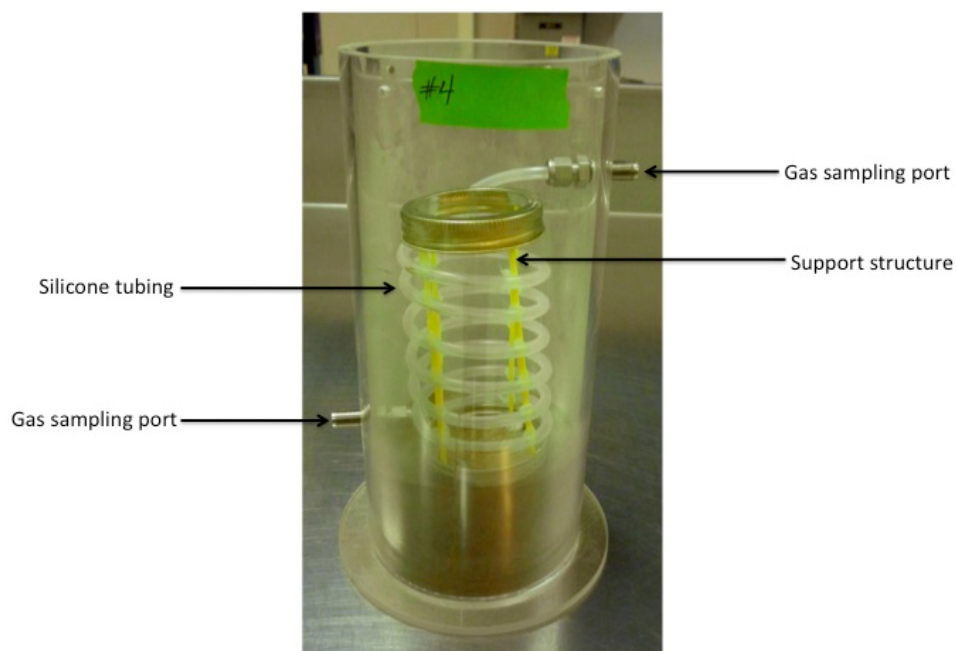


Figure 3.1. A photograph of one of the acrylic pots and silicone tubing coil used for rhizosphere gas sampling. The support structure holds the tubing in a coil and prevents collapse once soil is added to the pot.

3.2.3.2 Pea seed inoculation

Pea seeds (cv. CDC Meadow) were surface sterilized and pre-germinated as described in Section 3.2.2.1. Immediately before planting, seeds were inoculated with one of the five *R. leguminosarum* strains or a sterile water control by evenly applying 1 mL of rhizobia culture or water to eight sprouted pea seeds (see Section 3.2.2.1). One sprouted pea seedling was planted into each acrylic pot and each inoculation treatment was replicated six times. Rhizobial cell concentrations applied at planting are reported in Table 3.3.

Table 3.3. Estimated *R. leguminosarum* population applied to seeds immediately prior to planting in the sand-soil mixture.

<i>R. leguminosarum</i> strain	HUP status	Rhizobia population at planting
		cfu seed ⁻¹
128C52	+	1.74×10^8
128C53	+	1.43×10^8
128C79	-	1.50×10^8
PJB5J1	-	1.08×10^8
B151	n/a	1.29×10^8

3.2.3.3 *Rhizosphere and surface soil gas sampling*

Rhizosphere gas samples were collected from the gas sampling ports located in the side of the acrylic pot (Figure 3.1) on a weekly basis. The silicone tubing was first flushed with ultra high purity (UHP) N₂ ensuring that the tubing was flushed with a minimum of two-volumes of N₂ to remove any gases that had accumulated since the previous sampling. The tubing was then allowed to equilibrate with the surrounding soil for 30 min, during which gases from the soil atmosphere diffused into the tubing. To collect gas samples, a 60 mL syringe filled with UHP N₂ was inserted into one sampling port and was used to replace the equivalent volume of the sample being drawn off using a 20 mL syringe inserted into the other sampling port (Figure 3.2). This prevented a vacuum from developing and potentially collapsing the tubing. For full details on preliminary studies conducted to determine equilibration time and rhizosphere sampling protocol see Appendix C.



Figure 3.2. A photograph of the rhizosphere gas sampling set up used to collect samples from silicone tubing. The syringe on the left-hand side was filled with UHP N₂ and replaced the sample volume drawn off by the syringe on the right-hand side of the photograph.

Surface gas samples were collected using a split-plate lid fitted with a self-sealing septum and vent. The split-plate lid fit around the stem of the plant, and was further sealed around the plant stem by applying Glad Press'n Seal™; a cling film that adheres to most surfaces and itself. The lid was set in place 30 min prior to sample collection and secured to the pot using rubber bands.

The headspace between the soil surface and the lid was sampled through the septum by drawing off a 20 mL sample using a syringe. Each gas sample was injected into a pre-evacuated 12 mL Exetainer vial and analyzed on a gas chromatograph for N₂O, H₂, CO₂, and O₂. Parameters used for gas sample analysis are described in Section 3.2.4. Rhizosphere and surface samples were collected once a week for seven weeks; i.e., from seedling emergence to late pod-filling.

3.2.3.4 Plant sampling

Fifty-five days after planting (DAP), the pea plants had reached the late pod-filling stage, at which time the pots were destructively sampled. Roots and nodules were washed then nodules were counted, and all belowground biomass was dried at 60°C and weighed. Aboveground plant material, including grain from pea pods, was harvested, dried at 60°C, separated, and weighed.

3.2.4 Gas sample analysis

All gas samples were analyzed for H₂, CO₂, and O₂ using a Varian CP-4900 Micro Gas Chromatograph (Varian, Walnut Creek, CA). The CO₂ was identified using a thermal conductivity detector (TCD). The column is a Poraplot U, 10-m long with a 0.32-mm i.d. The detection limit is 80 ppm. Hydrogen and O₂ were identified using a TCD attached to a molecular sieve column that was 10-m long. The detection limit for H₂ was estimated to be <1 ppmv.

Nitrous oxide was analyzed on a Varian CP-3800 GC (Varian, Walnut Creek, CA) equipped with two electron capture detectors (ECD) and Poraplot Q coated plot-fused silica (0.32 mm i.d. × 10 m, with a 0.32 µm film thickness). Gas samples (300 µL) were introduced on-column using a split injection system (split ratio = 10) with Ar/CH₄ (P5: 95% Ar/5% CH₄) as the carrier gas and UHP He as the make-up gas. The lower detection limit for N₂O was determined to be 60 ppbv.

3.2.5 Up-scaling H₂ production from field pea

Up-scaling H₂ produced from HUP⁺ legume nodules to the field level provides an opportunity to compare different legume crops. A theoretical H₂ exposure rate (HExR; µmol H₂ cm⁻³ h⁻¹) of soil adjacent to legume nodules can be calculated using Equation 3.1 (Dong and Layzell, 2001), which takes into account the amount of N₂ a legume can fix during a growing season (NF; µmol N₂ ha⁻¹ season⁻¹) and assumes an electron allocation coefficient (EAC) of 0.67. Season length

(SL; h season⁻¹) is the time during the season in which N₂ fixation is active; depth of nodules (D; cm) is the depth below ground surface at which nodules are concentrated; row length (L; cm ha⁻¹) is the total length of all rows in a hectare; and width (W; cm) is the total cross-sectional width of the diffusion zone (Dong and Layzell, 2001). Dong and Layzell (2001) calculated HExRs based on the assumption that the H₂ diffusion zone extended 1, 2 or 4 cm outward from the nodule (i.e., W = 2, 4 or 8 cm).

$$\text{HExR} = [(3\text{NF} \times (1-\text{EAC})) / (\text{EAC} \times \text{SL})] / (\text{D} \times \text{L} \times \text{W}) \quad [3.1]$$

Dong and Layzell (2001) developed Eqn. 3.1 to estimate the amount of H₂ evolved from HUP soybean; thus, the parameters used in the equation were based on soybean planting practices. That is, the NF value was based on a soybean crop that fixed 200 kg N ha⁻¹ season⁻¹ and a season length of active fixation of 24 h day⁻¹ for 60 days (1440 h season⁻¹); the depth of nodules (D) was 10 cm with a total row length of 2 × 10⁶ cm ha⁻¹ (200 rows ha⁻¹ × 100 m row⁻¹); three exposure distances (W = 2, 4, or 8 cm) were investigated.

In this study, the parameters used in Eqn. 3.1 were selected to represent agricultural practices associated with growing pea in Saskatchewan. Three N fixation values also were selected based on BNF values measured from pea grown at Scott, SK (Farrell et al., 2011). The measured BNF values from cropping years 2008, 2009 and 2010 were compiled and the minimum (75 kg N ha⁻¹ season⁻¹), maximum (179 kg N ha⁻¹ season⁻¹) and mean (123 kg N ha⁻¹ season⁻¹) values selected for NF and converted to units of μmol N₂ ha⁻¹ season⁻¹. The calculated season length (SL = 896 h season⁻¹) was based on 16 hours of daylight over 56 days of active fixation (Jensen, 1987). Whereas the depth (D) of nodules was kept at 10 cm, row spacing for pea plants (22.5 cm) is narrower than soybeans, thus there are 444 rows ha⁻¹ (Farrell et al., 2011) yielding a total row length of 4.44 × 10⁶ cm ha⁻¹. The theoretical H₂ exposure rate was then calculated using the W values suggested by Dong and Layzell (2001). Using these values and Eqn. 3.1, the HExRs for field pea were estimated to range from 12 to 119 nmol H₂ cm⁻³ soil h⁻¹ (Table 3.4).

3.2.6 Statistical analysis

All experiments were set up using a completely randomized design; all statistical analyses were performed using CoStat for Macintosh ver. 6.400 (CoHort, 2008). Data transformations,

when necessary, were selected by process of elimination in order to obtain a normal distribution and homogeneity of variance (Field, 2005); all values reported in the text were back-transformed where required.

Table 3.4. The calculated hydrogen exposure rates (HExRs) for soil influenced by pea inoculated with HUP⁺ rhizobia. Three rates of BNF and three distances from the nodule (W) were used to show the range of HExR found in soil based on Equation 3.1.

Biologically fixed N	Total width (W) of H ₂ exposure	HExR
kg N ha ⁻¹ season ⁻¹	cm	nmol H ₂ cm ⁻³ h ⁻¹
75	2	50
75	4	25
75	8	12
123	2	82
123	4	41
123	8	20
179	2	119
179	4	59
179	8	30

Significant treatment (rhizobial) effects on H₂ production from roots and nodules, rhizosphere N₂O, surface N₂O, number of nodules, nodule weight, root weight, seed weight and shoot weight were assessed using analysis of variance (ANOVA). Treatment means were compared using the least significant difference (LSD) test at a 0.05 significance level; the LSD test was chosen because it is capable of analyzing uneven replicates due to missing values.

Hydrogen concentration data obtained under ambient atmospheric conditions were transformed using a reciprocal transformation. Hydrogen concentration data obtained under the H₂-enriched atmosphere (and reflecting H₂ production or consumption) were transformed using a square root transformation. Treatment differences for each time interval were assessed using a one-way ANOVA. Linear regression analysis was conducted to determine H₂ production rates from pea roots and nodules exposed to both ambient and H₂-enriched atmospheres.

Both rhizosphere N₂O concentrations and surface N₂O flux from the greenhouse study were transformed prior to statistical analysis. Rhizosphere N₂O concentrations were transformed using a logarithmic function and analyzed with a one-way ANOVA for each time interval. Surface N₂O production was analyzed using the same statistical tests, but following a reciprocal transformation of the gas flux data.

In addition to the ANOVA, correlations between the rhizosphere and surface N₂O results were evaluated using a Pearson Product Moment Correlation. Nitrous oxide production in the soil is thought to be associated with O₂, CO₂ and H₂ in the rhizosphere (Dong and Layzell, 2001; Golding and Dong, 2010); therefore, relationships between rhizosphere N₂O and rhizosphere O₂, CO₂ and H₂ also were assessed using the Pearson Correlation.

Statistical analyses were completed for all belowground and aboveground plant growth parameters: number of nodules, nodule weight, root weight, seed weight and shoot weight. All plant growth parameters were analyzed using a one-way ANOVA. Two plant growth parameters were transformed to reduce heterogeneity of variance and obtain a normal distribution: the number of nodules was transformed using a square root transformation, whereas root weight was transformed logarithmically.

The Kruskal-Wallis test, a non-parametric equivalent to a one-way ANOVA, was used to analyze data that were not normally distributed and did not have homogeneity of variance. The Kruskal-Wallis test does not make assumptions about the distribution of the data and analyses are conducted on ranked data (CoHort, 2001). The Kruskal-Wallis test was used to assess treatment effects on N₂O production from roots and nodules under ambient and H₂-enriched atmospheres, as well as on cumulative surface N₂O from the greenhouse study.

3.3 Results

3.3.1 Ambient and H₂-enriched atmosphere study

3.3.1.1 Hydrogen production from roots and nodules

Roots and nodules from pea inoculated with the HUP⁻ rhizobia strains (128C79 and PJB5J1) and exposed to the ambient air atmosphere yielded increasing H₂ concentrations over time (Figure 3.3). Conversely, the control treatments and the roots and nodules of pea inoculated with the HUP⁺ rhizobia exhibited near-zero H₂ production rates (Figure 3.3). At each sampling interval, significant ($P \leq 0.001$) differences in H₂ production from pea roots and nodules were detected among the six inoculant treatments. Indeed, roots and nodules grown with the HUP⁻ strains (128C79 and PJB5J1) produced significantly more H₂ at each sampling time than roots and nodules grown with HUP⁺ strains or the control treatments (Table 3.5).

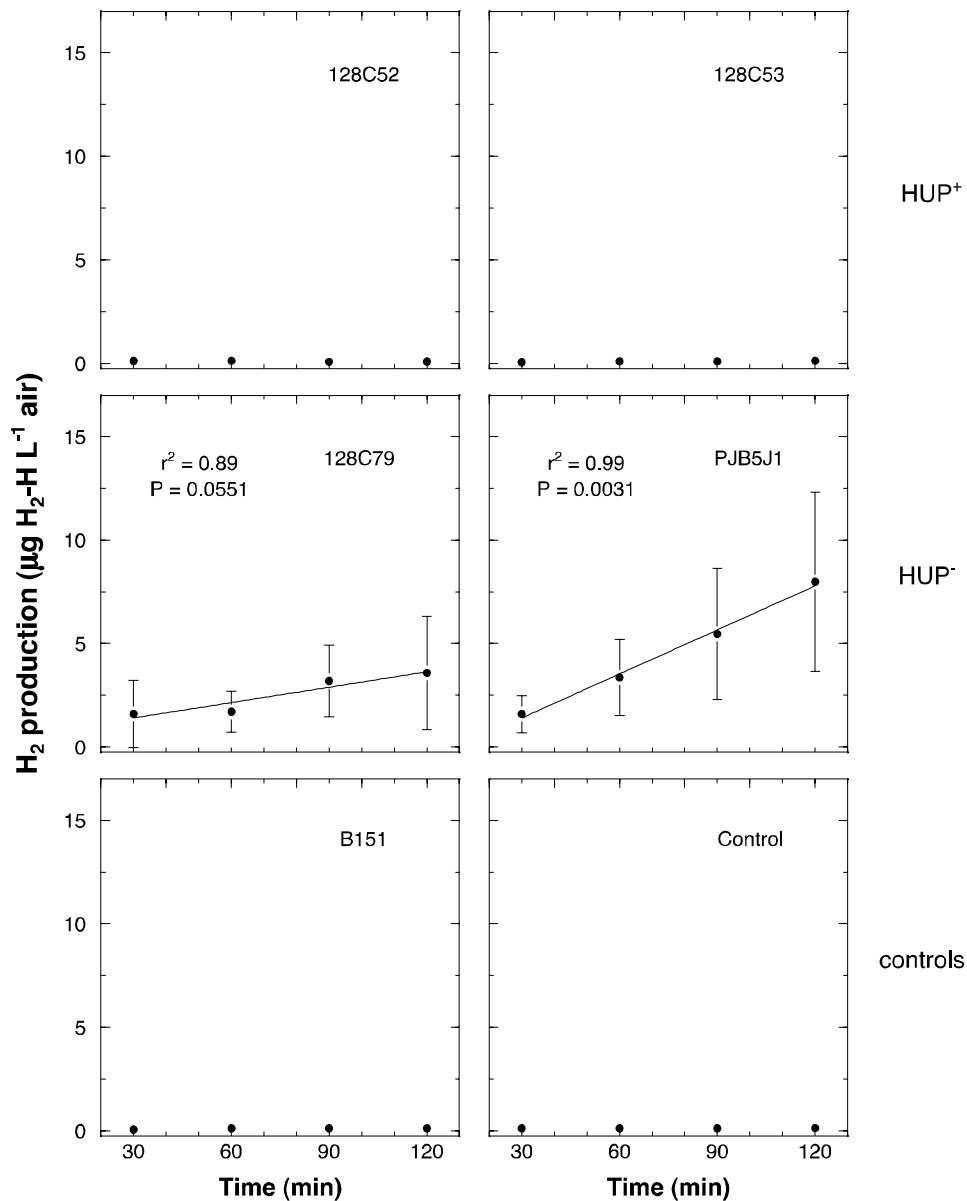


Figure 3.3. Mean H_2 production from pea roots and nodules inoculated with one of five *R. leguminosarum* strains or sterile water (Control). Plants were grown for four weeks in Leonard jars and excised root systems exposed to an ambient air atmosphere. Error bars are the standard deviation of the mean (n = 4).

Table 3.5. Net production/consumption of H₂ by excised roots and nodules of pea inoculated with one of four rhizobial strains differing in HUP status or a non-nodulating rhizobium, and a sterile water control. Roots and attached nodules were excised from four week-old pea plants and enclosed in a sealed atmosphere of ambient or H₂-enriched air (n = 4[¶]).

Treatment	HUP status	Time			
		30 min	60 min	90 min	120 min
<div> <div></div> <div>net production/consumption of H₂ (μL L⁻¹)</div> <div></div> </div>					
Ambient air atmosphere					
128C52	+	1.10 b [‡]	1.17 b	1.00 c	1.03 c
128C53	+	1.00 b	1.13 b	1.15 bc	1.29 b
128C79	-	5.88 a	9.30 a	16.67 a	17.64 a
PJB5J1	-	11.43 a	26.67 a	41.67 a	50.00 a
B151	n/a	1.01 b	1.19 b	1.17 b	1.06 bc
Control	n/a	1.04 b	1.05 b	1.10 bc	1.28 bc
LSD _{0.05}		4.29 [§]	5.46 [§]	7.00 [§]	4.86 [§]
H ₂ -enriched atmosphere					
128C52	+	-8.10	-15.74 c	-11.62 c	-24.62 [¶] c
128C53	+	-22.72	-13.05 c	-11.53 c	-13.53 c
128C79	-	9.79	20.98 b	30.15 b	40.25 b
PJB5J1	-	29.96 [¶]	54.35 a	77.80 a	94.48 a
B151	n/a	-5.48	-3.65 c	-6.03 c	-13.57 c
Control	n/a	-8.35	-14.24 c	-9.50 [¶] c	-28.17 c
LSD _{0.05}		ns [‡]	1.33	2.63 [§]	3.83 [§]

[†] Within columns, means followed by the same letter are not significantly different according to LSD_{0.05}.

[‡] ns denotes no significant differences.

[§] LSD results are conservative estimates where some treatments had n < 4 due to missing values.

[¶] n = 3; gas samples were lost due to a GC malfunction.

The roots and nodules of pea inoculated with the HUP⁻ rhizobia and exposed to the H₂-enriched atmosphere (Figure 3.4) yielded results similar to those observed when the roots and nodules were exposed to an ambient air atmosphere; i.e., H₂ production increased as the exposure time increased. Moreover, under a H₂-enriched atmosphere, net H₂ production from roots inoculated with HUP⁻ rhizobia was almost double that produced under an ambient atmosphere (Table 3.5). Conversely, H₂ appeared to be consumed in the jars containing the roots and nodules of pea inoculated with the HUP⁺ rhizobia particularly for strain 128C52 (Figure 3.4). Hydrogen also was consumed in the jars containing the roots of pea that were either uninoculated (sterile water control) or inoculated with the non-nodulating rhizobium B151.

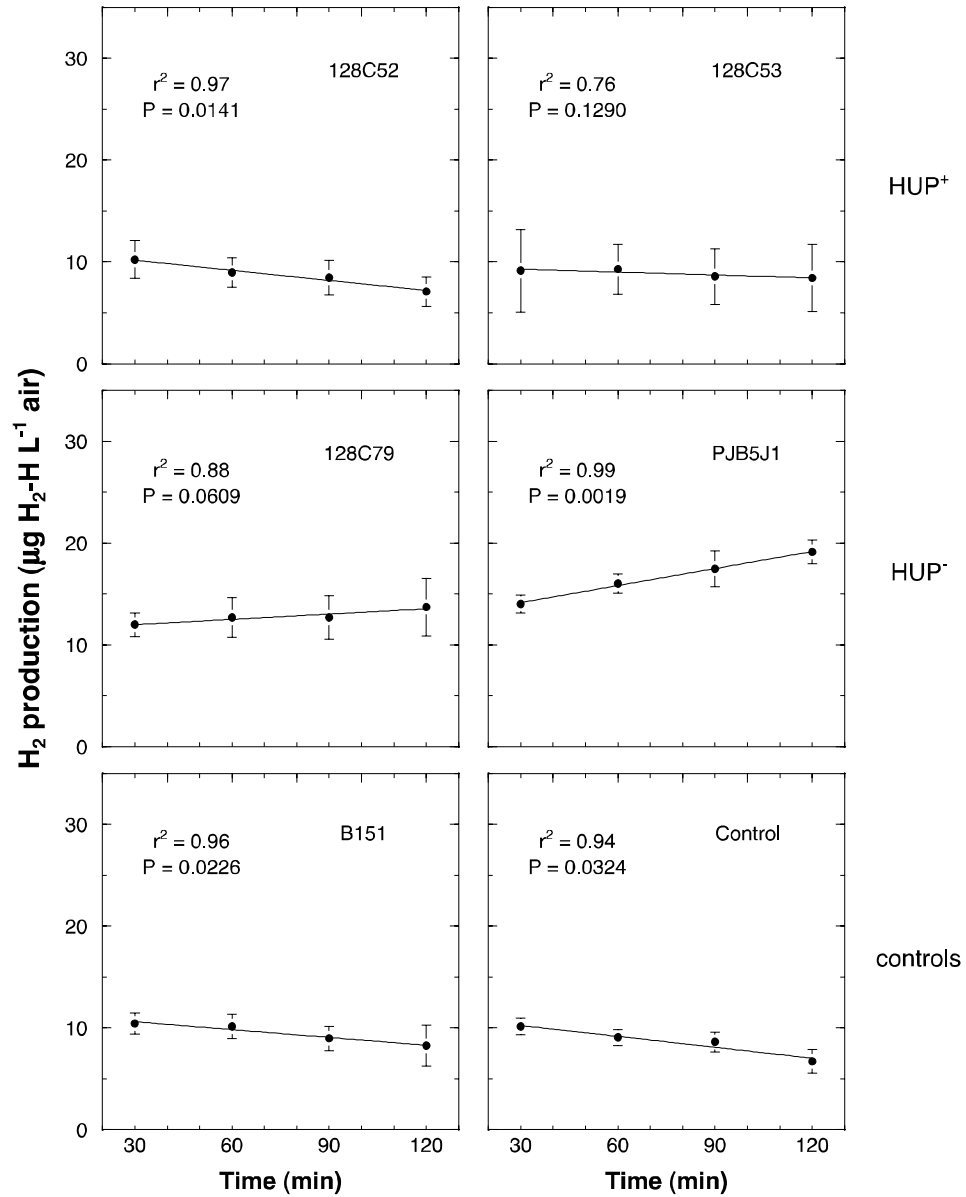


Figure 3.4. Mean H_2 production from pea roots and nodules inoculated with one of five *R. leguminosarum* strains or sterile water (Control). Plants were grown for four weeks in Leonard jars and excised roots systems exposed to a H_2 -enriched atmosphere. Error bars are standard deviation of the mean ($n = 4$).

In the jars containing a H₂-enriched atmosphere, changes in total H₂ concentration were often obscured by the high background concentration (100 ± 7 ppmv). Consequently, blanks (i.e., jars containing the H₂-enriched atmosphere, but no roots or nodules) were included in the experiment and net H₂ production/consumption was determined by subtracting the H₂ concentration measured in the blanks from the total H₂ concentration in the jars containing the roots and nodules (Table 3.5). Significant ($P \leq 0.001$) differences in H₂ production occurred at all time intervals after the first 30 min (Table 3.5). Excised root systems inoculated with the HUP⁻ strains, 128C79 and PJB5J1, produced significantly more H₂ than the root systems inoculated with HUP⁺ strains and control treatments.

3.3.1.2 Nitrous oxide production from roots and nodules

Nitrous oxide production by pea roots and nodules exposed to an ambient air or H₂-enriched atmosphere was measured at the same time as H₂ production. Under the ambient air atmosphere, the roots and nodules had no impact on N₂O concentrations; i.e., N₂O concentrations did not differ from the background concentration measured in the blanks (Figure 3.5). Likewise, the roots and nodules of pea inoculated with the various rhizobia showed no increase in N₂O production when exposed to the H₂-enriched atmosphere (Figure 3.6).

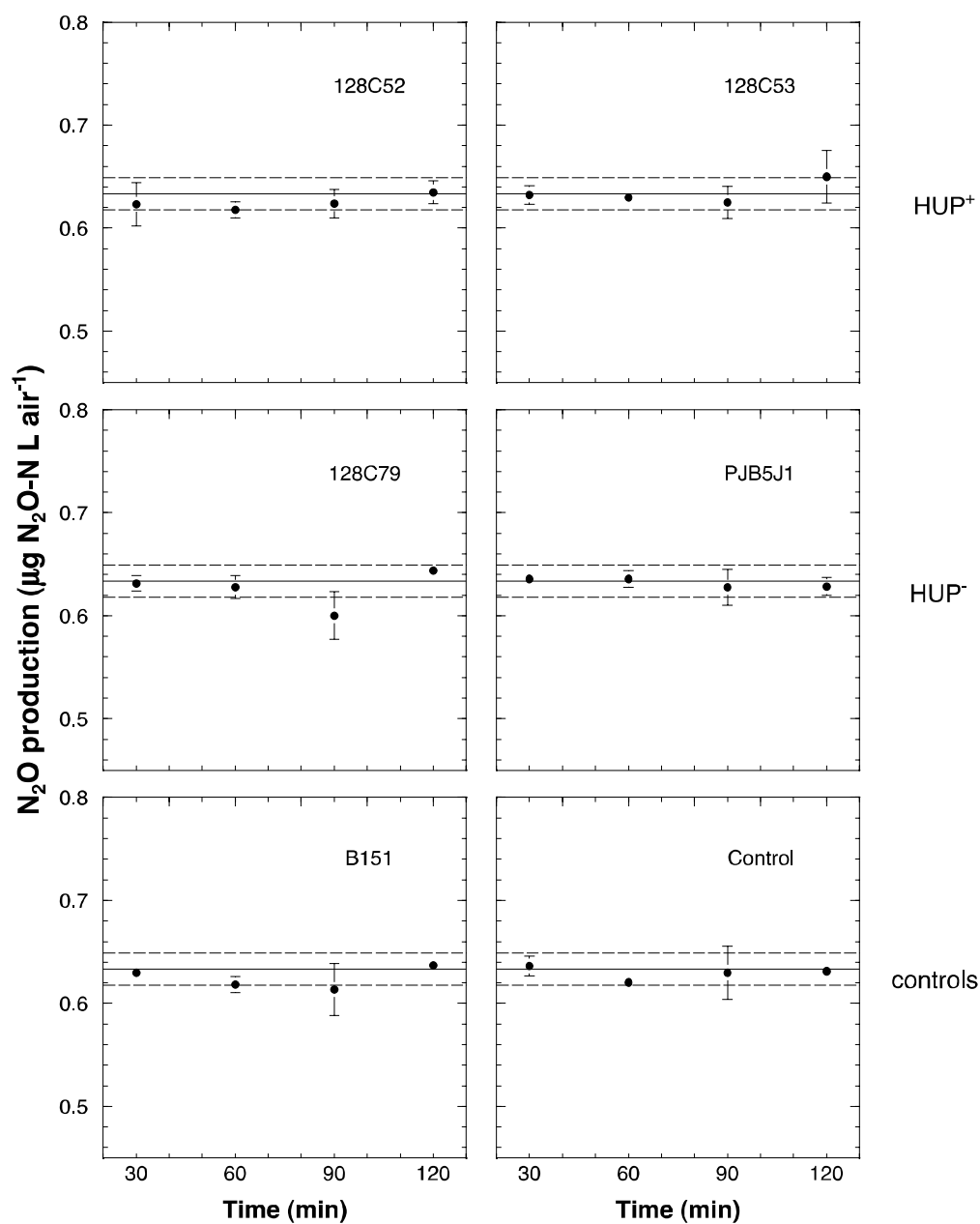


Figure 3.5. Mean N_2O concentrations measured from pea roots and nodules under an ambient air atmosphere. Solid horizontal lines represent mean N_2O in blank media jars. Dashed horizontal lines are the mean blank value \pm the standard deviation of the blank media jars. Error bars are standard deviation of the mean at each time interval ($n = 4$).

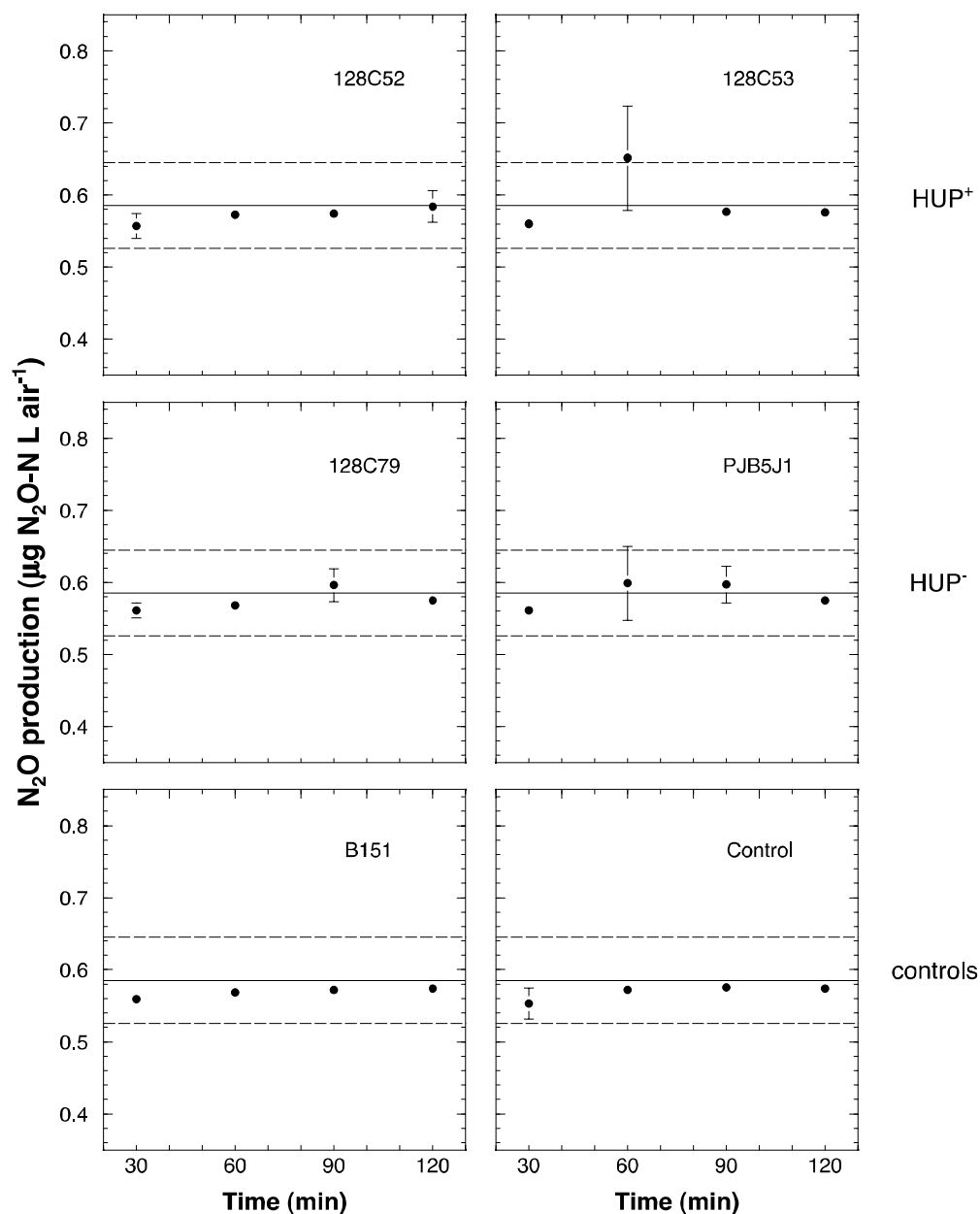


Figure 3.6. Mean N_2O concentrations measured from pea roots and nodules under a H_2 -enriched atmosphere. Solid horizontal lines represent mean N_2O in blank media jars. Dashed horizontal lines are the mean blank value \pm the standard deviation of the blank media jars. Error bars are standard deviation of the mean at each time interval ($n = 4$).

3.3.2 Greenhouse pot study

3.3.2.1 Rhizosphere and surface soil N₂O production

In general, N₂O concentrations in the rhizosphere (Figure 3.7) and emissions at the soil surface (Figure 3.8) peaked between 13 and 27 DAP, declined and then leveled off to near-zero production and emissions by DAY 53. The box and whisker plots presented in Figures 3.7 and 3.8 provide a visual representation of the variability and range of the data among replicates for individual inoculation treatments. Nitrous oxide concentrations exhibited a greater range in the rhizosphere of pea inoculated with the HUP⁺ rhizobia (128C52 and 128C53) and control treatments (B151 and Control) than in the rhizosphere of pea inoculated with the HUP⁻ rhizobia (128C79 and PJB5J1) (Figure 3.7). Nitrous oxide emissions from the soil columns exhibited similar temporal patterns (Figure 3.8) and, overall, there was a strong positive correlation ($r = 0.844$; $P \leq 0.001$) between the N₂O concentrations in the rhizosphere and the N₂O flux at the surface. Moreover, a strong positive correlation between N₂O concentrations and emissions was detected for each of the six treatments (Table 3.6).

Differences in the amounts of N₂O produced in the rhizosphere were significant only at 34 DAP and 41 DAP (Table 3.7) reflecting that on these days the variability between replicates was small. At 34 DAP, the mean N₂O concentration in the rhizosphere of pea was 4× to 21× greater than ambient and decreased in the order: 128C79 > uninoculated control > B151 > 128C53 ≈ 128C52 > PJB5J1 (Table 3.8). At 41 DAP, the mean N₂O concentration in the uninoculated control was *ca.* 12× ambient and significantly ($P \leq 0.05$) greater than any of the inoculated pea treatments (which ranged from *ca.* 3-5× ambient) (Table 3.8). Significant differences between the various inoculant treatments, however, were not observed. Likewise, there were no treatment effects on the surface N₂O flux on any of the sampling days (Table 3.7).

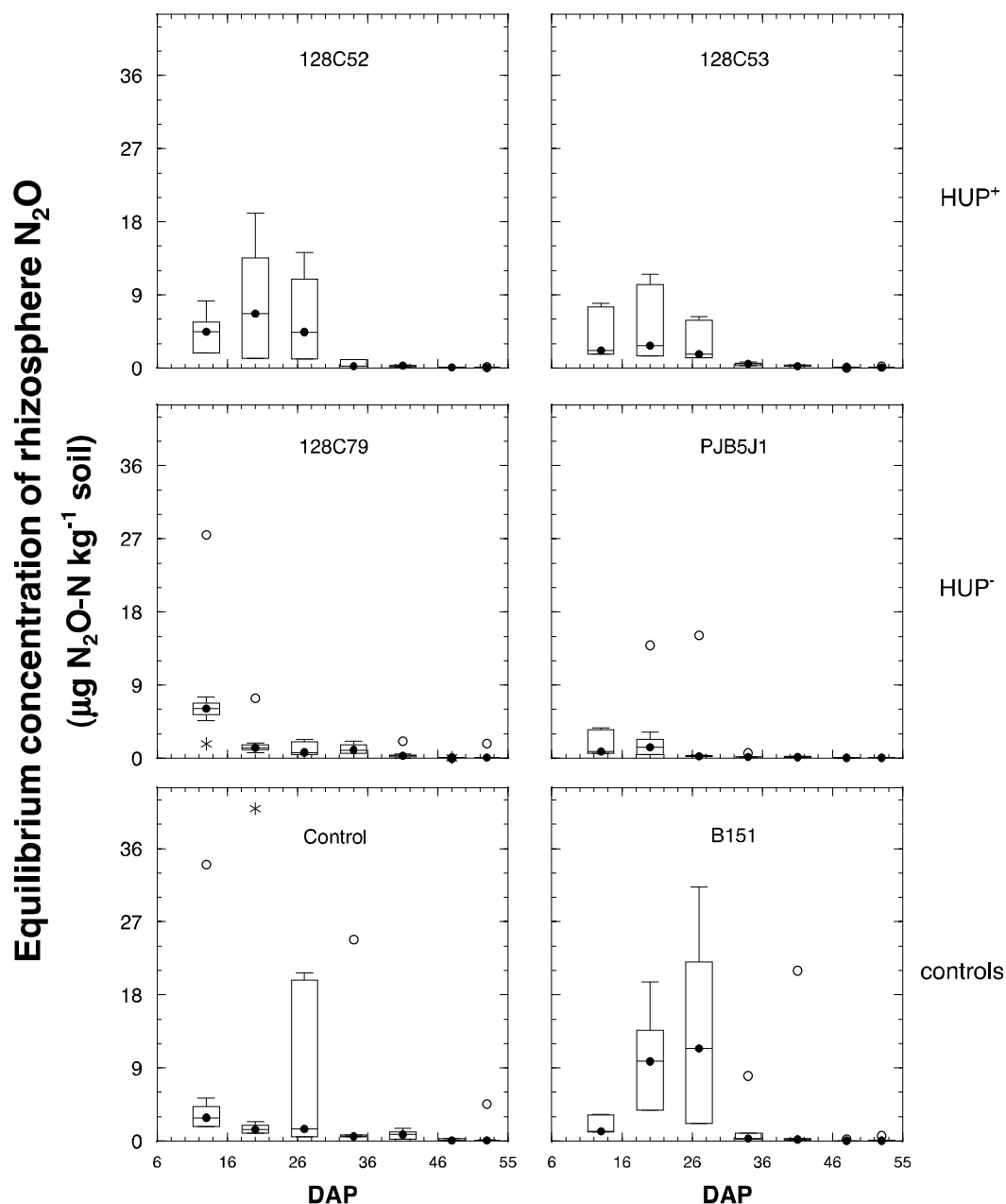


Figure 3.7. N_2O concentration in the rhizosphere of pea inoculated with HUP⁺, HUP⁻, and non-nodulating rhizobia and an uninoculated control. Concentrations were measured weekly from seedling emergence (13 DAP) to late pod-filling (53 DAP). The box component of the box and whisker plots is composed of the 75th, 50th (median) and 25th percentile and the upper whisker is the median plus the inter-quartile range. Moderate outliers are represented as * and extreme outliers are shown as °. Note: the ambient N_2O concentration ($0.35 \mu L N_2O L^{-1}$) was equivalent to $0.047 \mu g N_2O-N kg^{-1} soil$).

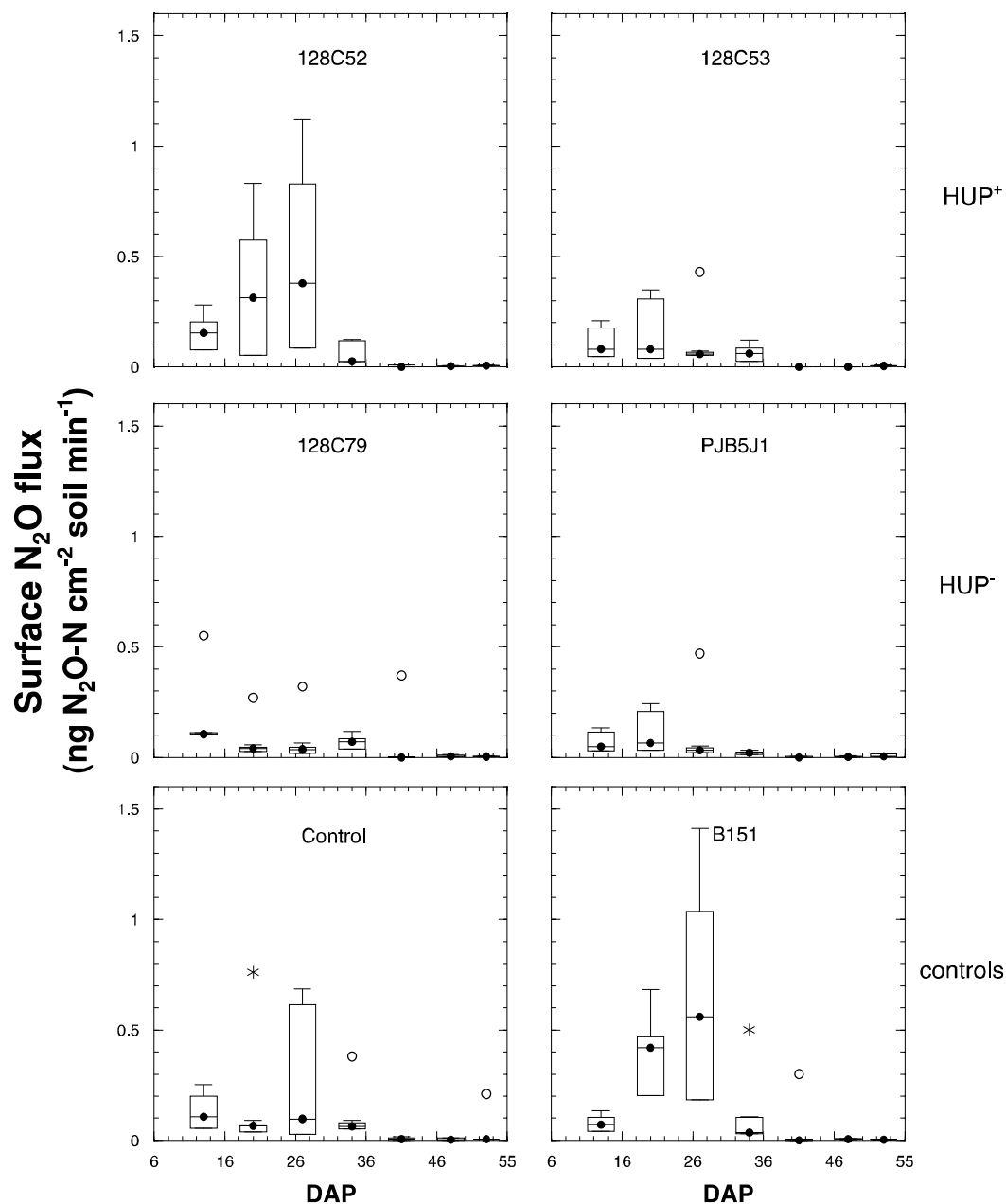


Figure 3.8. N_2O emissions from soil columns growing pea inoculated with HUP^+ , HUP^- , and non-nodulating rhizobia and an uninoculated control (sterile water). Production was measured weekly from seedling emergence (13 DAP) to late pod-filling (53 DAP). The box component of the box and whisker plots is composed of the 75th, 50th (median) and 25th percentile and the upper whisker is the median plus the inter-quartile range. Moderate outliers are represented as * and extreme outliers are shown as °.

Table 3.6. Pearson Correlation analysis between rhizosphere N₂O concentration ($\mu\text{g N}_2\text{O-N kg}^{-1}$ soil) and surface N₂O flux ($\mu\text{g N}_2\text{O-N cm}^{-2} \text{ min}^{-1}$) from a greenhouse pot study. The correlation coefficient, *r*, was determined for each rhizobial treatment.

Treatment	n	HUP status	Pearson correlation coefficient	S.E. of <i>r</i>
128C52	39	+	0.903	0.071
128C53	42	+	0.763	0.102
128C79	42	-	0.796	0.096
PJB5J1	42	-	0.966	0.041
B151	42	n/a	0.918	0.063
Control	42	n/a	0.930	0.060

Table 3.7. Statistical significance for rhizosphere and surface soil N₂O concentrations ($\mu\text{L L}^{-1}$) collected weekly from pea seedling emergence to late pod-filling. A one-way ANOVA for each weekly sampling event was used to determine differences in N₂O production (*n* = 6).

Days after planting	Degrees of Freedom	Rhizosphere N ₂ O	Surface N ₂ O
		Probability	
13	5	0.2182	0.4962
20	5	0.2245	0.2989
27	5	0.1027	0.2017
34	5	0.0446 *	0.2002
41	5	0.0305 *	0.7972
48	5	0.0845	0.3221
53	5	0.1121	0.5852

*Indicates significance at the 0.05 probability level.

Table 3.8. Equilibrium N₂O concentrations ($\mu\text{L L}^{-1}$) in the rhizosphere of pea on 34 and 41 DAP. Significant differences in N₂O concentrations were observed only on these two days. Values are the mean concentrations of six replicates sampled after 30 min. Note: the ambient N₂O concentration was $0.35 \mu\text{L L}^{-1}$ (which is equivalent to $0.047 \mu\text{g N}_2\text{O-N kg}^{-1}$ soil).

Treatment	HUP status	Days after planting (DAP)	
		34	41
		rhizosphere N ₂ O ($\mu\text{L L}^{-1}$)	
128C52	+	2.83 bc [†]	1.58 b
128C53	+	3.45 abc	1.88 b
128C79	-	7.19 a	1.91 b
PJB5J1	-	1.26 c	1.08 b
B151	n/a	4.08 abc	1.77 b
Control	n/a	5.46 ab	4.25 a
LSD_{0.05}		1.28[§]	0.77[§]

[†] Within columns, means followed by the same letter are not significantly different according to LSD (0.05).

[§] LSD results are conservative estimates where some treatments had *n*<6 due to missing values.

3.3.2.2 Cumulative N₂O emissions at the soil:atmosphere interface

Cumulative N₂O emissions at the soil:atmosphere interface were greatest during the period from 13 DAP to 27 DAP (Figure 3.9A), with 72% \pm 4% of total emissions occurring during this period. Emission rates during this period ranged from about 1.2 ng N₂O-N cm⁻² d⁻¹ from soils planted to pea inoculated with HUP⁻ rhizobia (128C79 and PJB5J1) or the HUP⁺ rhizobium 128C53 to 2.1 ng N₂O-N cm⁻² d⁻¹ from soils planted to pea inoculated with the HUP⁺ rhizobium 128C52 or the sterile water control and 3.1 ng N₂O-N cm⁻² d⁻¹ from soils planted to pea inoculated with the non-nodulating rhizobium B151. Although treatment differences in cumulative N₂O emissions were not significant (Figure 3.9B), there remained a significant correlation between the mean N₂O concentration in the rhizosphere (averaged across time for each treatment) and cumulative N₂O emissions ($r = 0.906$, $P = 0.13$).

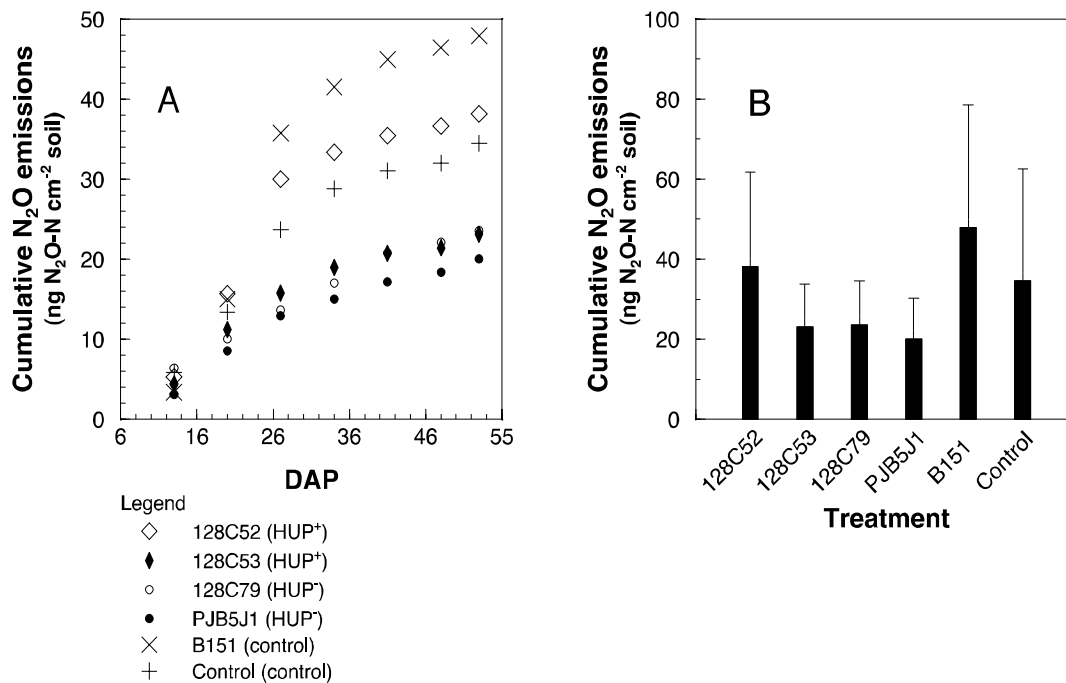


Figure 3.9. Cumulative N₂O emissions from soil columns of pea inoculated with HUP⁺, HUP⁻, and non-nodulating rhizobia and an uninoculated control. Nitrous oxide emissions were measured from seedling emergence to late pod-filling. Error bars in panel B are the standard deviation of the mean for each rhizobial treatment.

3.3.2.3 Additional rhizosphere gas samples

In addition to N₂O concentrations, O₂ consumption and the production of CO₂ and H₂ also were measured in the rhizosphere (see Figures 3.10–3.12). In all treatments, O₂ concentrations in the rhizosphere were lower than those in the ambient atmosphere (i.e., <21%) indicating O₂ consumption. Rhizosphere O₂ concentrations were greatest ($17 \pm 1\%$) at seedling emergence (13 DAP) and decreased to a relatively steady state ($12 \pm 0.5\%$) by 20 DAP (Figure 3.10). Carbon dioxide concentrations in the soil atmosphere followed a similar temporal pattern (Figure 3.11), but whereas O₂ was being consumed, CO₂ was being produced via root and microbial respiration. As a result, rhizosphere CO₂ concentrations were generally two-orders of magnitude greater than ambient concentrations [ranging from *ca.* 2 to 10% (v/v)]. A Pearson Correlation between surface and rhizosphere CO₂ concentrations was also conducted; however, there was no significant correlation noted even though surface and rhizosphere N₂O concentrations showed a strong positive correlation. Measureable concentrations of H₂ were found in the soils on all sampling dates, and for all treatments (Figure 3.12). However, H₂ concentrations in the rhizosphere did not vary among the inoculation treatments. At the same time, changes in H₂ concentrations in the soil atmosphere followed temporal patterns that were similar to those observed for O₂ consumption and CO₂ production.

Correlations between N₂O concentrations in the rhizosphere and rhizosphere concentrations of H₂ were generally very weak ($r < 0.30$) and were not significant (Table 3.9). Correlations between rhizosphere concentrations of N₂O and CO₂ also were weak ($r < 0.45$), though significant ($P \leq 0.01$) correlations were detected in the rhizosphere of pea inoculated with the HUP⁺ strains 128C79 and PJB5J1. Surprisingly, there also was a significant positive correlation ($r = 0.509$; $P \leq 0.001$) between N₂O and O₂ concentrations in the rhizosphere of pea inoculated with HUP⁺ strain, 128C79 (Table 3.9).

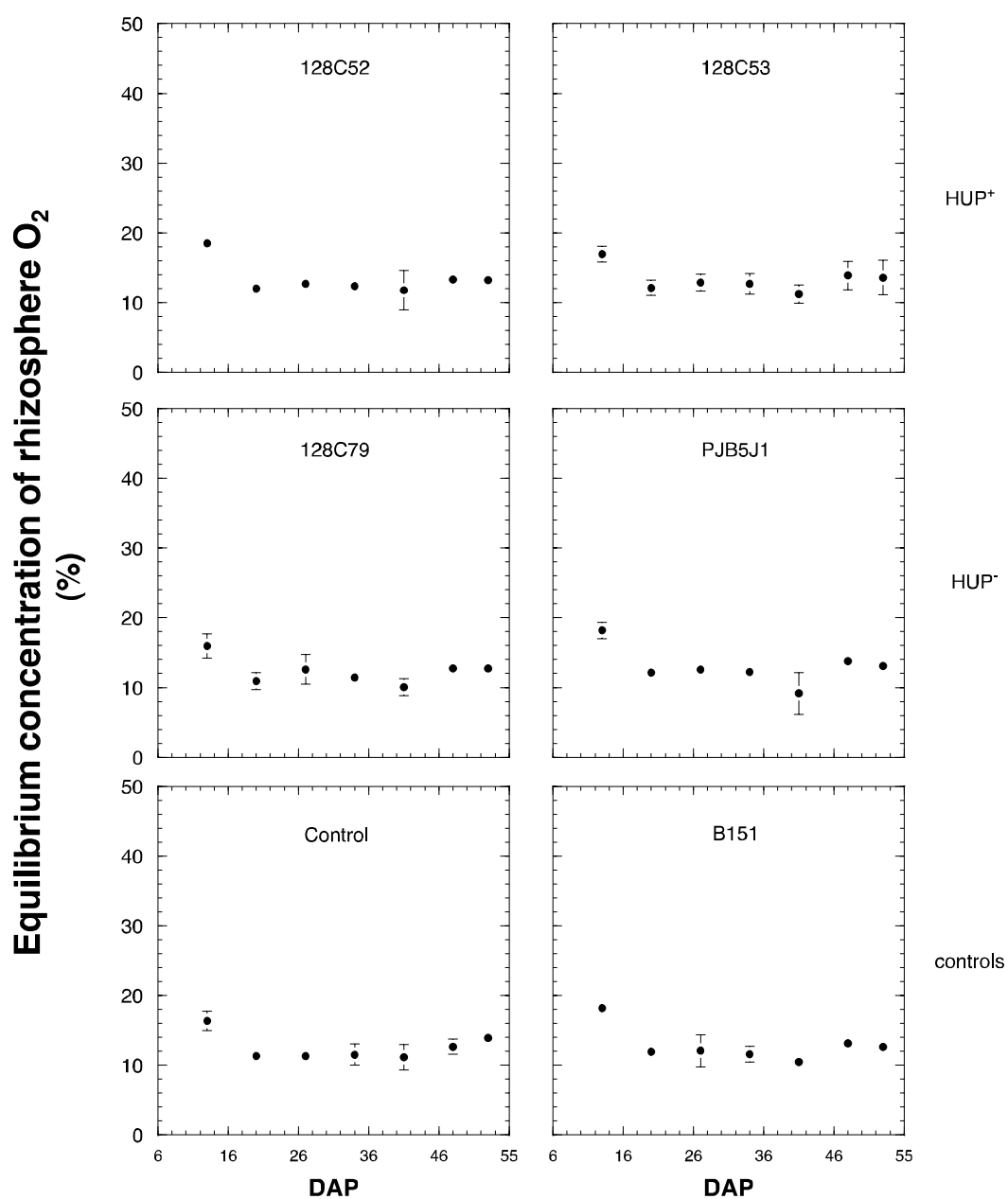


Figure 3.10. Rhizosphere O_2 concentration in pea measured weekly from seedling emergence to late pod-filling in a greenhouse pot study. Values are the mean O_2 concentration for six replicates for each pea inoculation treatment (HUP^+ , HUP^- , control). Error bars show the standard deviation of each mean.

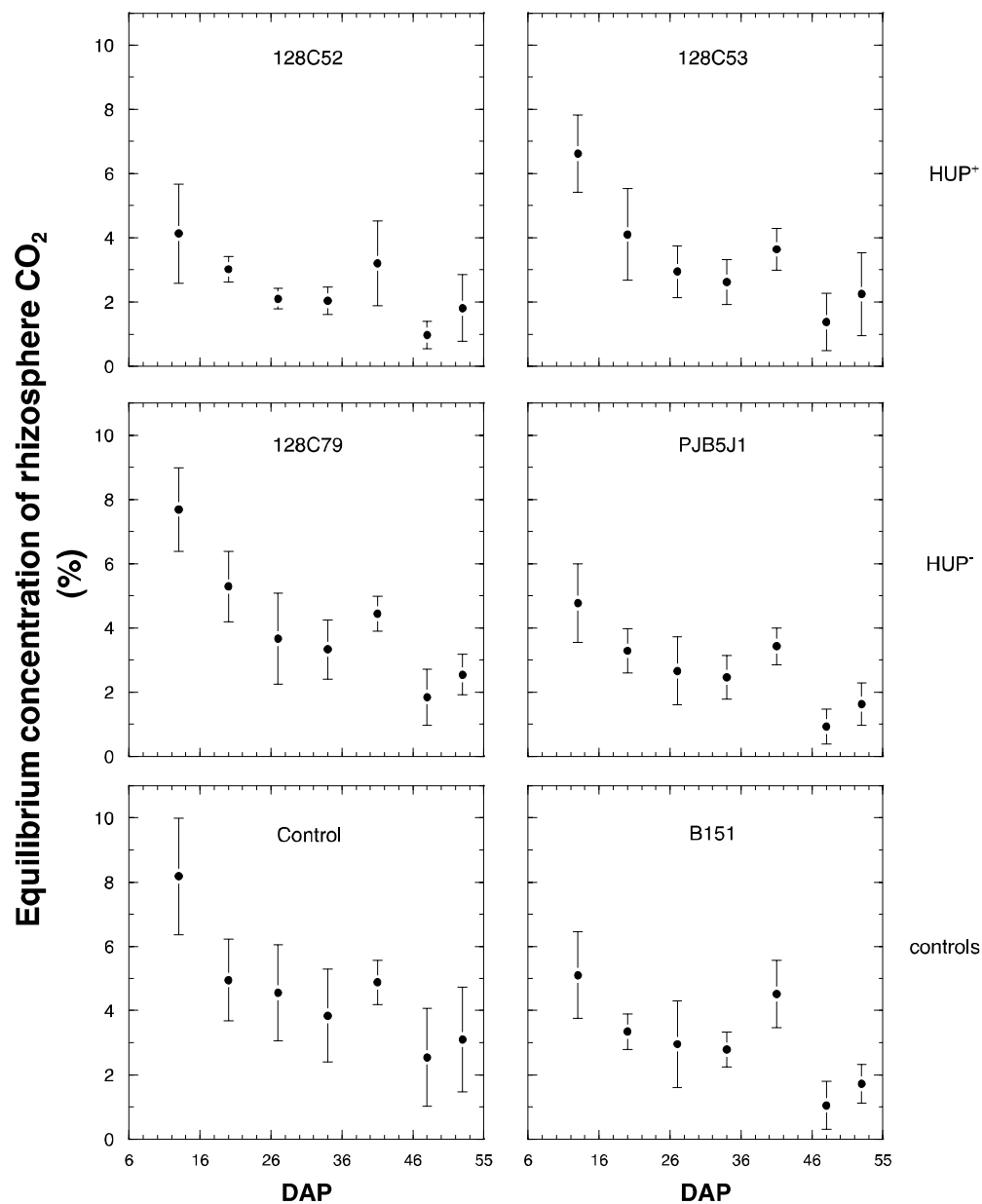


Figure 3.11. Rhizosphere CO₂ concentrations in pea measured weekly from seedling emergence to late pod-filling in a greenhouse pot study. Values are the mean CO₂ concentrations for six replicates for each pea inoculation treatment (HUP⁺, HUP⁻, control). Error bars show the standard deviation of each mean.

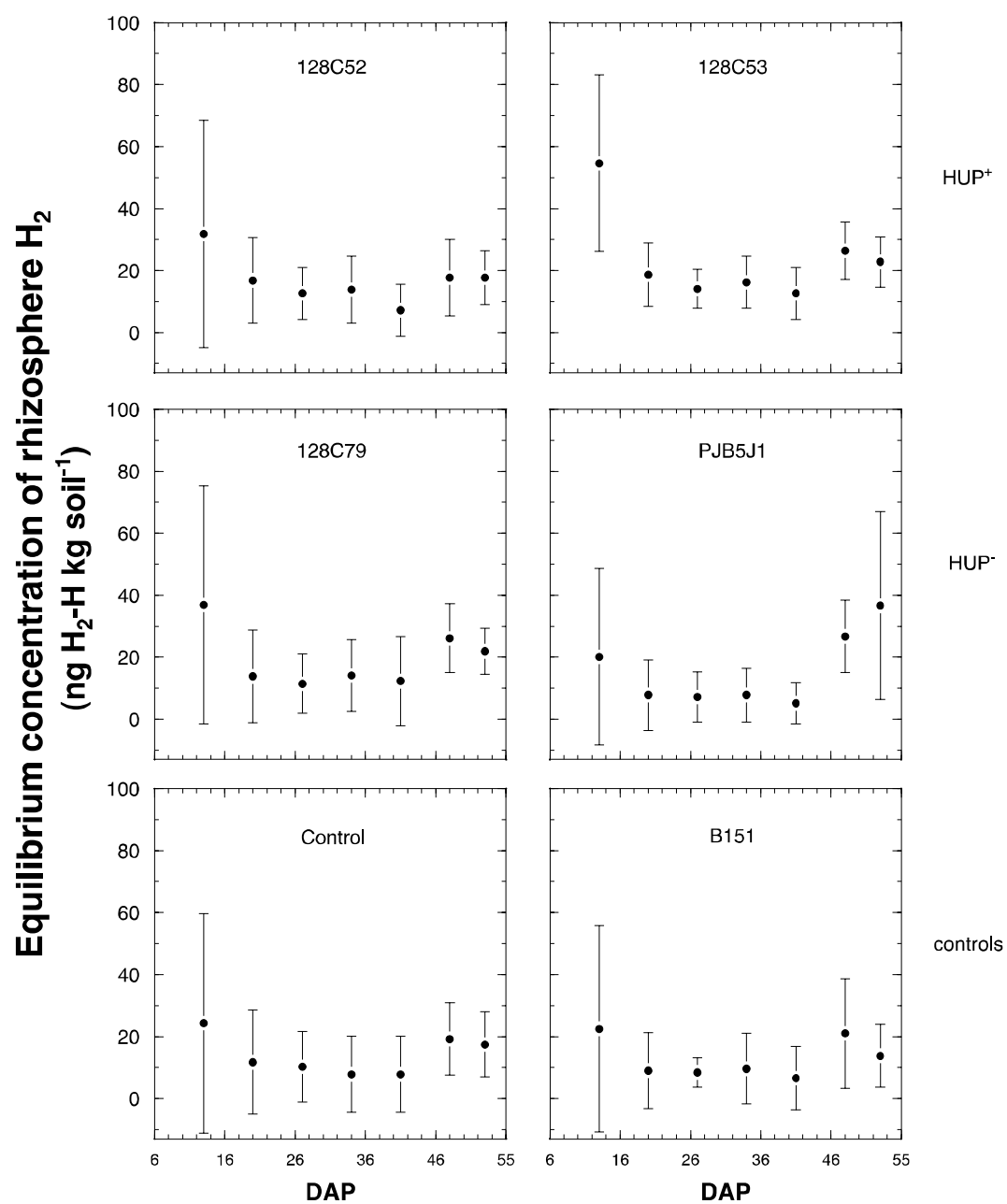


Figure 3.12. Rhizosphere H_2 production in pea measured weekly from seedling emergence to late pod-filling in a greenhouse study. Values are the mean H_2 production for six replicates for each pea inoculation treatment (HUP⁺, HUP⁻, control). Error bars show the standard deviation for each mean.

Table 3.9. Pearson Product Correlation Coefficient, r , for correlations between rhizosphere N_2O and rhizosphere H_2 , CO_2 , and O_2 concentrations from a greenhouse pot study. Correlations were analyzed by pea inoculation treatment.

Treatment	HUP status	Rhizosphere N_2O correlated with -		
		Rhizosphere H_2	Rhizosphere CO_2	Rhizosphere O_2
		r		
128C52	+	0.039	0.268	0.021
128C53	+	0.258	0.273	0.156
128C79	-	-0.028	0.416 **	0.509 ***
PJB5J1	-	-0.149	0.410 **	0.045
B151	n/a	-0.076	0.074	-0.005
Control	n/a	-0.081	0.255	-0.041

, * indicates significance at the 0.01, and 0.001 probability level, respectively.

3.3.2.4 Belowground plant material

Pea inoculated with control treatments (i.e., the non-nodulating rhizobium B151 and the sterile water control) were not expected to form nodules on the roots; however, when the plants were harvested, nodules were present on some of the roots. Presumably, there was a small population of indigenous rhizobia (HUP status unknown) in the soil, which infected and formed nodules on the roots of some of the control plants. Regardless, there were significantly fewer and smaller nodules present on the roots of the control plants than on the roots of the pea inoculated with HUP⁺ and HUP⁻ rhizobia (Figure 3.13A and B).

Plants with the most nodules included those inoculated with the HUP⁻ rhizobia (128C79 and PJB5J1) and the HUP⁺ rhizobium 128C52 (Figure 3.13A). However, whereas these rhizobia produced small, single-lobed nodules, pea inoculated with the HUP⁺ 128C53 strain exhibited large, multi-lobed nodules. Thus, although pea inoculated with 128C53 had relatively few nodules, the total weight of the nodules was quite large ($0.1109 \text{ g plant}^{-1}$; Figure 3.13B). Indeed, the average total weight of the 128C53 nodules was comparable to (i.e., not significantly different from) that of the plants inoculated with the 128C52, 128C79 and PJB5J1 rhizobia (Figure 3.13B). Moreover, the total weight of nodules recovered from roots inoculated with the HUP⁺ and HUP⁻ strains was significantly greater than that of the nodules recovered from the controls (i.e., non-nodulating rhizobium B151 and sterile water treatments).

There was no clear trend in the effect of inoculation on the production of root biomass. Indeed, belowground biomass (root) production was greatest for pea inoculated with the HUP⁺ rhizobium 128C52 and the non-nodulating rhizobium B151 (Figure 3.13C). Plants yielding the

least amount of root biomass included the uninoculated control and pea inoculated with the HUP⁻ rhizobium 128C79.

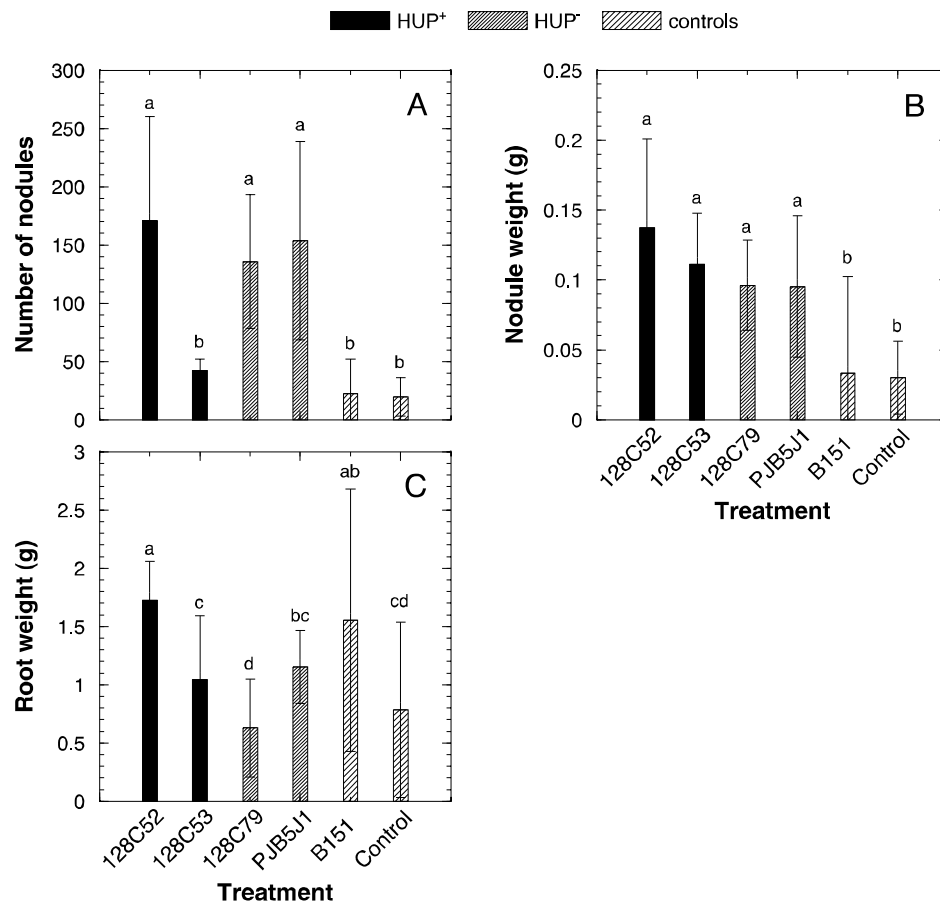


Figure 3.13. Belowground plant growth parameters for pea grown under greenhouse conditions: nodule count (A), nodule weight (B) and root weight (C). All plant measurements were made using dried plant material collected at harvest 55 (DAP). Bars represent the mean values ($n = 6$); error bars are the standard deviation for each mean. Different letters indicate significant differences ($P \leq 0.05$) between means.

3.3.2.5 Aboveground plant material

Treatment (inoculant) differences were observed in the aboveground biomass (i.e., shoot and seed) production (Figure 3.14). Seed dry weights were significantly ($P \leq 0.01$) greater in pea inoculated with the two HUP⁺ strains than in the pea inoculated with either control treatment. Indeed, field pea inoculated with the control treatments B151 and sterile water (Control) had the

lowest seed dry weights compared to pea inoculated with either the HUP⁺ or HUP⁻ rhizobia, even though the HUP⁻ treatments were not significantly different (Figure 3.14A). Pea inoculated with the HUP⁻ rhizobium, PJB5J1, also yielded significantly higher seed weights than the uninoculated control treatment.

Pea inoculated with the HUP⁻ rhizobium 128C79, HUP⁺ rhizobium 128C53, or sterile water (uninoculated control) produced less shoot biomass than the other inoculation treatments (Figure 3.14B). Pea inoculated with the HUP⁺ strain 128C52 and HUP⁻ strain PJB5J1 had the highest dry shoot weights. Pea inoculated with non-nodulating rhizobium B151 also had significantly greater shoot weights than the sterile water control.

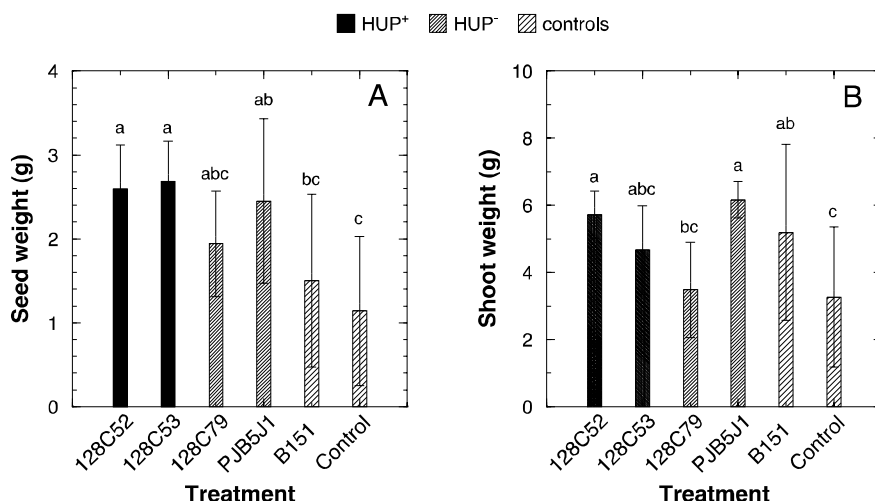


Figure 3.14. Aboveground plant growth parameters for pea grown under greenhouse conditions: seed weight (A) and shoot weight (B). All biomass measurements were made from dried plant materials collected at harvest (55 DAP). Bars represent the mean values (n = 6); error bars are the standard deviation of each mean. Different letters indicate significant differences ($P \leq 0.05$) between means.

3.3.3 Field scale H₂ production from pea

Using the experimental parameters described in Equation 3.1, the theoretical H₂ exposure rate (HE_{ExR}) was up-scaled to the field level. That is, assuming a season length of 56 days with 16 hours of N₂ fixation each day, a pea field could produce between 90,000 and 214,000 L H₂ ha⁻¹ season⁻¹ at STP. At the mean N-fixation rate of 123 kg N ha⁻¹ season⁻¹, a pea field would produce 147,000 L H₂ ha⁻¹ season⁻¹. Full calculation details are presented in Appendix D.

3.4 Discussion

Hydrogen is an obligate by-product of the nitrogenase reaction and is produced during the conversion of atmospheric N_2 to NH_4^+ . Legumes infected with rhizobia produce H_2 during BNF; however, the presence or absence of the HUP enzyme determines whether this H_2 is recycled by the rhizobia (Evans et al., 1987). When the rhizobia are HUP^+ (i.e., the enzyme is present), very little, if any H_2 escapes the rhizobium–nodule system and a portion of the energy expended during H_2 production is recovered by recycling the H_2 (Robson and Postgate, 1980). However, when rhizobia are HUP^- (i.e., the enzyme is absent), the H_2 produced escapes the nodule and diffuses into the surrounding soil (Lafavre and Focht, 1983; Robson and Postgate, 1980).

The methylene blue reduction assay was used to confirm the HUP status of the four nodulating rhizobia used in this study, strains 128C52 and 128C53 were confirmed as HUP^+ and strains 128C79 and PJB5J1 were confirmed as HUP^- . Hydrogen evolution from roots and nodules under ambient air demonstrated that the HUP^+ strains were capable of recycling nearly all the H_2 evolved with very little H_2 leaving the nodule. Conversely, the HUP^- strains produced significantly more H_2 than either the HUP^+ or control inoculation treatments. Significantly higher H_2 concentrations from HUP^- rhizobia compared to HUP^+ rhizobia also have been observed in previous studies (Nelson and Child, 1981; Peoples et al., 2008). Similar results were observed from pea roots and nodules placed under a H_2 -enriched atmosphere; i.e., H_2 concentrations were greater in jars containing roots inoculated with HUP^- rhizobia. Moreover, under the H_2 -enriched atmosphere, the production of H_2 from HUP^- nodules was almost double that measured under ambient atmospheric conditions. As well, under the H_2 -enriched atmosphere, the plant roots inoculated with HUP^+ rhizobia appeared to consume H_2 ; i.e., H_2 concentrations decreased with time. Similar results were observed for the control treatments, suggesting that pea roots or microorganisms associated with the roots (e.g., endophytic or rhizoplane microorganisms) may be capable of consuming H_2 .

Nitrous oxide production and BNF are important N transformations that have been studied both independently and in conjunction with one another. There are a number of pathways in which BNF and N_2O production may be connected, such as direct denitrification by rhizobia (O'Hara and Daniel, 1985), N-rich plant residue decomposition (Ellert and Janzen, 2008), or nitrification of biologically fixed N (Galloway, 1998). This study examined only N_2O emissions

from actively fixing field pea nodules.

The incubated field pea roots and nodules did not produce N_2O , even though H_2 production from the HUP^- treatments indicated that nodules were actively fixing N_2 . This suggests that there was no direct link between BNF and N_2O production regardless of the HUP status of the rhizobia. That is, the HUP^- field pea nodules produced no more N_2O than the HUP^+ or control treatments, even when the atmosphere in the incubation jars was enriched with H_2 . However, given that there was no soil and hence, no soil microbial communities associated with the roots, this was not totally unexpected. However, similar results were observed when field pea inoculated with HUP^+ and HUP^- rhizobia were grown in soil. For example, whereas significant amounts of N_2O were being produced in the rhizosphere (i.e., N_2O concentrations were 4–20 \times greater than ambient), treatment (inoculant) effects were generally quite small and were not significant.

In a survey of studies involving N_2O emissions associated with legumes, Rochette and Janzen (2005) suggested that even though growing legumes can produce substantial amounts of N_2O , it is likely not produced directly from BNF. Furthermore, N_2O production from nodules was associated with degraded, decomposing nodules and not from active nodules (Inaba et al., 2009). Similarly, higher rates of denitrification in soil from N_2 -fixing faba beans (*Vicia faba*) (Kilian and Werner, 1996), and higher total N_2O -N emissions from legume crops compared to non-legumes over a growing season (Ghosh et al., 2002) are likely from N compounds released in root exudates during the growing season or decomposition of N-rich residues and not directly from BNF (Rochette and Janzen, 2005).

The lack of N_2O produced from the field pea roots and nodules reported here is further support that the five strains selected are likely not capable of direct denitrification. Certain rhizobial strains are capable of denitrification and N_2O production (O'Hara and Daniel, 1985). However, not all rhizobia are able to produce N_2O in pure culture or in symbiosis with legume roots (Zhong et al., 2009). In the five *R. leguminosarum* strains selected, BNF and direct denitrification by rhizobia did not produce N_2O under the experimental conditions (i.e., N_2O concentrations did not differ from background levels).

Extensive work quantifying the amount of H_2 evolved from HUP^- soybean nodules has focused on artificially treating soil with H_2 at rates similar to those evolved from soybean nodules (Dong and Layzell, 2001; Dong et al., 2003). Comparing artificially H_2 -treated soil with

air-treated soil revealed the reducing power of H_2 was linked to increased O_2 consumption and CO_2 fixation by H_2 -oxidizing microbial communities in the soil (Dong and Layzell, 2001). Hydrogen-treated soils had greater CO_2 fixation rates than control soils suggesting that soil around HUP^- nodules would also have greater CO_2 fixation rates (Stein et al., 2005). The increased rates of microbial O_2 consumption and CO_2 fixation were thought to create conditions in the soil that favour denitrification and N_2O production by generating hypoxic or anoxic zones (Golding and Dong, 2010). Indeed, preliminary tests reported H_2 -treated soils produced a tenfold increase in N_2O compared to air-treated soils (Golding and Dong, 2010). In the present study, however, field pea inoculated with HUP^+ and HUP^- rhizobia and grown in soil showed no difference in N_2O concentrations measured in the rhizosphere and had no significant effect on the N_2O flux at the soil:atmosphere interface even though the pea roots and nodules of plants inoculated with the HUP^- rhizobial strains produced significantly more H_2 . As well, correlations between the concentrations of H_2 , O_2 and CO_2 in the rhizosphere and the concentrations of N_2O in the rhizosphere were quite low ($r \leq 0.509$), even though the literature suggests that this should not be the case. Indeed, N_2O production associated with HUP^- legumes is implicated with H_2 production, O_2 consumption and CO_2 fixation occurring in the soil surrounding legume nodules (Dong and Layzell, 2001; Golding and Dong, 2010; Stein et al., 2005). This suggests that there should be positive correlations between H_2 and N_2O concentrations in the rhizosphere, as well as a negative correlation between N_2O and O_2 and CO_2 concentrations in the rhizosphere. In the present study, no significant correlations between rhizosphere H_2 and N_2O concentrations were detected; however, this could presumably be because microbial communities consumed H_2 quickly and it could not be measured accurately. On the other hand, there was a significant positive correlation between rhizosphere CO_2 and N_2O in pea inoculated with both HUP^- strains; though, pea inoculated with the HUP^- rhizobia did not produce the greatest amount of N_2O . Based on the theory that H_2 in the rhizosphere increases CO_2 fixation by soil microorganisms (Stein et al., 2005) and favours denitrification and N_2O production (Golding and Dong, 2010), a negative correlation would have been expected. However, because plants were grown and soil was not artificially treated with H_2 , root respiration produced CO_2 and that is what was measured in the rhizosphere. Microbial CO_2 fixation was not measured directly and it is likely the process was overwhelmed by the amount of CO_2 produced by roots. In addition, there was a positive correlation between rhizosphere O_2 and N_2O in pea inoculated with HUP^- strain 128C79, but not

with the HUP⁻ strain PJB5J1, even though N₂O concentrations in the pea rhizosphere were lowest in the HUP⁻ treatments. Hydrogen-oxidizing bacteria are thought to mediate the association between rhizosphere H₂ from HUP⁻ legumes and rhizosphere CO₂, O₂, and N₂O (Dong and Layzell, 2001; Golding and Dong, 2010; Maimaiti et al., 2007), but microbial communities were not investigated in this study and warrant future investigation.

There are several possible explanations for the lack of N₂O produced by the pea inoculated with HUP⁻ rhizobia. Much of the work done to date has focused on artificially treating soil with H₂ and looking at gas exchange without the presence of plant roots in the soil (Dong and Layzell, 2001; Golding and Dong, 2010). Plant roots are important in establishing the rhizosphere, which can be rich in microbial activity. For example, microbial community structure was different in soil adjacent to HUP⁻ soybean nodules compared to H₂-treated soil even though the variation in the community structure was stimulated by H₂ (Zhang et al., 2009). The only difference between the two soils was the presence of soybean roots in the soil adjacent to HUP⁻ nodules. Roots presumably impact the rhizosphere soil in ways that cannot be simulated solely by H₂-treating bulk soil.

Another possible explanation could be the difference in H₂ produced by various legumes. Artificial H₂ soil treatment is based on the rate of H₂ evolution from soybean nodules. A soybean crop, that fixes 200 kg N ha⁻¹ in a season, was calculated to produce 240,000 L H₂ ha⁻¹ season⁻¹ (Dong et al., 2003). A similar value, 215,000 L H₂ ha⁻¹ season⁻¹, was also estimated from other soybean measurements (Peoples et al., 2008). However, using the same equation but adjusting the parameters to reflect pea production in Saskatchewan, seasonal H₂ production was estimated at only 147,000 L H₂ ha⁻¹ indicating that H₂ production in pea is 32% to 39% lower than that in soybean. Field pea does not typically fix as much N₂ through BNF as soybean and therefore does not produce as much H₂. This could mean the effects attributed to H₂ exposure in soil under soybean could be more pronounced than those from H₂ production under pea. The lack of correlation between rhizosphere N₂O, CO₂ and O₂ for pea inoculated with HUP⁻ rhizobia also suggests that the reducing power associated with H₂ in the soil was not evident in the greenhouse study.

The ability to detect treatment differences in N₂O production from field pea was, at least in part, a reflection of the high variability in N₂O production between replicates. High variability in soil N₂O emissions has been noted in other studies and it is often attributed to “hot spots” in the

soil (Ellert and Janzen, 2008). It is difficult to account for hot spots, and soil was homogenized as much as possible prior to potting. Increased replication may be needed in future studies.

Recent studies have suggested that part of the non-N benefits of planting legumes may be attributable to a H_2 fertilization effect. Microorganisms in the rhizosphere soil oxidize H_2 released by HUP^- nodules (Dong and Layzell, 2001), altering the microbial community structure, which in turn can influence plant growth (Dong et al., 2003; Zhang et al., 2009). Soil exposed to a H_2 enriched atmosphere at a rate similar to that evolved from soybean was shown to promote plant growth in legume crops and subsequent non-legume crops (Dong et al., 2003). Soybean, barley, canola and wheat all had increased root and shoot dry weight when grown in soils that had been pre-treated with H_2 (Dong et al., 2003). These increases in plant growth were attributed to increases in H_2 -oxidizing microorganisms in the soil around the nodules although microbial communities were not investigated in that study (Dong et al., 2003). Similar results also were reported in barley yields from plants grown on plots previously planted with HUP^- or HUP^+ soybean (Dean et al., 2006). Grain yield from barley on HUP^- soybean stubble was significantly higher than grain yield from barley grown after HUP^+ soybean (Dean et al., 2006). These studies were concerned with subsequent crops following HUP^+ or HUP^- treatment or a H_2 pre-treatment and did not compare the plant growth differences between HUP^+ and HUP^- soybean.

Plant growth increases observed from soil surrounding HUP^- nodules or H_2 -treated soil is accredited to H_2 -oxidizing bacteria (Maimaiti et al., 2007). Bacteria capable of oxidizing H_2 were isolated from soil and several of the isolates acted as plant growth-promoting rhizobacteria (PGPR) stimulating primary root growth in wheat and total plant growth in *Arabidopsis* (Maimaiti et al., 2007). Hydrogen-treated soil had significantly higher microbial activity, biomass and differences in bacterial community structure compared to air-treated soil (Stein et al., 2005; Zhang et al., 2009). Although, in the present study, bacteria capable of oxidizing H_2 were not investigated, several plant growth parameters were measured to determine if there were differences between field pea inoculated with HUP^+ or HUP^- rhizobia. The plant growth parameters measured included: number of nodules on pea roots, nodule weight, root weight, seed weight and shoot weight. The only differences in the field pea were the inoculation treatments applied at planting. In general, pea inoculated with HUP^+ and HUP^- rhizobia had a higher number of nodules, total nodule weight and seed weight than pea inoculated with control treatments. However, pea inoculated with HUP^- rhizobia 128C79 had low root and shoot

weights. In general, pea inoculated with a nodulating strain of *R. leguminosarum* capable of fixing N_2 , regardless of HUP status, showed greater growth than pea inoculated with a non-nodulating (control) treatment. This suggests that plant growth differences are the result of access to additional fixed N instead of a H_2 fertilization effect.

Much of the work done on the H_2 fertilization effect has focused on a subsequent rotation following a legume rotation or plants grown on soil pre-treated with H_2 to simulate a previous HUP⁻ legume rotation. The plant growth promoting benefits may not be apparent in the legume phase itself (Peoples et al., 2008), but may appear in the crop following a legume phase or H_2 pre-treatment (Dean et al., 2006; Dong et al., 2003; Maimaiti et al., 2007). If a subsequent non-legume crop had been grown on the soil from the greenhouse study, there may have been measurable differences in plant growth. Plant growth promoting properties associated with H_2 diffusing into the soil were not observed in soybeans inoculated with HUP⁻ rhizobia (Peoples et al., 2008) even though the nodules produced similar amounts of H_2 to those reported by another study that did find differences in plant growth (Dong et al., 2003). The HUP⁻ rhizobia in the soybean nodules produced substantially more H_2 compared to the HUP⁺ rhizobia and uninoculated soybean. However, there were no differences in dry shoot weight or in seed yield between HUP⁺ and HUP⁻ soybean (Peoples et al., 2008). These results are similar to what was observed in the HUP⁺ and HUP⁻ inoculation treatments for field pea.

3.5 Conclusion

Hydrogen production associated with nodules from pea inoculated with HUP⁻ rhizobia was significantly greater than that associated with pea inoculated with HUP⁺ rhizobia, a non-nodulating rhizobia or an uninoculated control. There was no N_2O production from actively fixing pea nodules regardless of HUP status or H_2 concentration around the nodules, indicating that pea nodules did not produce N_2O directly through BNF. When pea roots and nodules were investigated within a soil profile, HUP⁻ rhizobia did not stimulate N_2O production even though H_2 production potentially creates conditions that may favour denitrification. As well, the plant growth promoting properties associated with H_2 stimulating microbial communities were not observed in HUP⁻ pea plants; however, the effect on a subsequent crop was not investigated.

4. SUMMARY AND CONCLUSIONS

It was hypothesized that H_2 gas, produced as a byproduct of biological nitrogen fixation (BNF) in field pea and diffusing into soil, would increase N_2O production and emission from the soil. In order to investigate this hypothesis, experiments were carried out in both the growth chamber and greenhouse. The first (growth chamber) experiment had two objectives: (i) determine whether actively fixing HUP^- pea nodules produced more H_2 than HUP^+ nodules and (ii) determine whether a H_2 -enriched atmosphere around pea nodules stimulated N_2O production. The objectives for the second (greenhouse) experiment were to determine (i) if pea inoculated with HUP^- rhizobia produced more N_2O than pea inoculated with HUP^+ rhizobia when grown in soil and (ii) if HUP^- rhizobia enhanced plant growth in field pea.

Under laboratory conditions, HUP^- pea nodules produced significantly more H_2 than HUP^+ nodules and control treatments. However, when pea roots and nodules were surrounded by a H_2 -enriched atmosphere to simulate the high H_2 concentration occurring around HUP^- nodules in the soil, there was no additional N_2O production (i.e., N_2O concentrations remained at ambient levels). The first experiment was conducted in the absence of soil; consequently, H_2 diffusing from the nodule could not interact with soil microbial communities that may have otherwise affected N_2O production. Hence, the second experiment built on the knowledge that HUP^- pea nodules produced substantially more H_2 than HUP^+ pea nodules and focused on growing pea plants in soil and measuring rhizosphere and surface soil N_2O production. The premise behind this was H_2 diffusing into the rhizosphere is taken up by H_2 -oxidizing bacteria and causes an increase in O_2 consumption and CO_2 fixation in the rhizosphere (Golding and Dong, 2010). Anoxic and hypoxic zones may develop due to increased O_2 consumption or microbial CO_2 fixation which could build carbon pools and provide reducing power, both conditions that favour denitrification and potentially N_2O production (Golding and Dong, 2010). However, pea inoculated with HUP^- rhizobia showed no increase in N_2O production compared to HUP^+ rhizobia or non-nodulating control treatments. In addition, plant growth parameters were assessed in the second experiment because previous studies with soybean had found that HUP^- rhizobia promoted plant growth compared to HUP^+ rhizobia (Dean et al., 2006; Dong et al., 2003). However, pea plant growth parameters, including aboveground and belowground measurements, showed no significant differences between HUP^+ and HUP^- rhizobia.

The results of these experiments do not support the hypothesis put forth at the beginning of the study, which was largely based on a preliminary report where a tenfold increase in N_2O production was observed from H_2 -treated bulk soil (Golding and Dong, 2010). There are several differences between the two studies that could account for these differences. First, actively growing pea plants inoculated with HUP⁻ rhizobia were used to treat the soil instead of using H_2 gas to treat bulk soil in the absence of plants. The presence of plant roots and a true rhizosphere could have affected the fate of the H_2 produced by HUP⁻ nodules. Furthermore, the soil used in this greenhouse experiment was different than the soil used in the bulk soil treatment reported by Golding and Dong (2010). The microbial communities could differ between the two soils as well as a number of physical soil properties that could all contribute to different results. To date, however, there have been no studies comparing how different soil types respond to both artificial H_2 treatment and/or legume crops. Finally, calculations in Appendix D show that on average, HUP⁻ pea nodules do not produce as much H_2 as HUP⁻ soybean nodules. Hydrogen evolution from soybean and pea inoculated with HUP⁻ rhizobia were not compared directly in this study, but the amount of H_2 diffusing into the soil may alter O_2 consumption and CO_2 fixation and the conditions that favour denitrification.

Saskatchewan is a Canadian leader in pulse production and has a strong agricultural sector. There has been a debate for some time as to whether BNF and N_2O emissions are connected since both are important N transformations (Rochette and Janzen, 2005). Since agriculture is responsible for approximately half of the N_2O emissions in Canada, it is important to inventory N_2O emissions (Helgason et al., 2005). This study supports recent research that indicates that BNF does not directly contribute to N_2O emissions. Hydrogen evolution from HUP⁻ *R. leguminosarum* strains did not appear to increase N_2O emissions; however, further work in this area is needed. This study provided foundational information that could be built upon. The next step could focus on microbial communities in different legume crops and different soils to determine if HUP⁻ rhizobia influence denitrifier communities and N_2O emissions under different conditions. As well, further work into the plant growth promotion in subsequent crops needs to be investigated. Only a pea crop was grown in the greenhouse experiment and no plant growth enhancements were observed; however, much of the work done with soybean has focused on a subsequent non-legume rotation. The lack of N_2O production from HUP⁻ pea in this study contrasts with that reported by Golding and Dong (2010), so further research is needed to

reconcile or explain these differences. Finally, more research needs to focus on legume crops other than soybean because soybean are not suited to the climate in western Canada and the mechanisms and pathways involved could differ between various legumes and under different climatic conditions.

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6. APPENDICES

APPENDIX A: CONFIRMING AND QUANTIFYING GROWTH OF RHIZOBIAL STRAINS

Objective

Preliminary experiments were conducted to confirm the selected bacterial strains as rhizobia and quantify the growth of each strain over time. Growth curves were created for each *Rhizobium* strain so that similar concentrations could be applied to pea seeds in subsequent experiments.

Materials and Methods

Five strains of *Rhizobium leguminosarum* were selected for use throughout the project. Strains 128C52, 128C53 and 128C79 were obtained from Dr. J. Germida's collection in the Department of Soil Science at the University of Saskatchewan (Saskatoon, SK, Canada); strains 3855PJB5J1 and B151 were obtained from Dr. M. Hynes in the Department of Biological Sciences at the University of Calgary (Calgary, AB, Canada). Strains 128C53, 3855PJB5J1 and B151 are isogenic strains: 128C53 is a known HUP⁺ strain and the parent strain of the other two; B151 is a non-nodulating mutant and 3855PJB5J1 is an isogenic HUP⁻ strain created by introducing a Sym plasmid into strain B151 (Dr. M. Hynes, person. comm.)

Congo Red Assay

The first diagnostic test was a congo red (diphenyl-bis- α -naphthylaminesulfonate) dye assay, which is a negative selection test for rhizobia (Hahn, 1966). Rhizobial colonies weakly absorb the congo red dye compared to other bacteria and appear translucent or white against the red growth medium (Somasegaran and Hoben, 1994). Congo red solution consists of 250 mg of congo red dye dissolved in 100 mL of distilled water. The assay was conducted by incorporating 10 mL of congo red solution into every 1.0 L of yeast mannitol agar (YMA); rhizobial cultures were then grown in yeast mannitol broth (YMB), with a 10⁻⁵ dilution series of each culture plated on YMA (Somasegaran and Hoben, 1994; Vincent, 1970). Yeast mannitol broth was prepared by dissolving 10.0 g of mannitol, 0.5 g of K₂HPO₄, 0.2 g of MgSO₄•7H₂O, 0.1 g of NaCl, and 0.5 g of yeast extract in 1.0 L of distilled water and adjusting the pH to 6.8 with HCl (Somasegaran and Hoben, 1994; Vincent, 1970). Yeast mannitol agar was prepared by adding 15 g of agar to

1.0 L of YMB before autoclaving (Somasegaran and Hoben, 1994).

Gram Stain

Rhizobia are Gram-negative bacteria; therefore, Gram stains also were used to screen the isolates and confirm their status as rhizobia (Somasegaran and Hoben, 1994). A Gram stain is a colormetric reaction that will remain violet-colored if the bacteria are Gram-positive or lose the violet color and turn red if the bacteria are Gram-negative (Somasegaran and Hoben, 1994). Rhizobia were grown on YMA spread plates until there were visible colonies, a loopful of each strain was heat fixed to a glass slide, and stained (Somasegaran and Hoben, 1994). Gram stain slides were observed under 10×, 40× and 100× magnification to observe bacteria cell morphology.

Growth Curves

Growth curves were obtained by plotting the optical density (OD) of freshly prepared bacterial suspensions versus time. Flasks of sterilized YMB were inoculated with a loopful of pure culture and incubated at room temperature on a rotary shaker at 100 rpm. Optical density readings for each strain were taken twice daily for the first two days of the incubation and then once a day for the third and fourth days. Optical density was measured using a Beckman DU[®] 650 spectrophotometer (Beckman Coulter, Inc., Brea, CA) calibrated with YMB and set to a 540 nm wavelength (Somasegaran and Hoben, 1994). The OD readings were subsequently converted to cell numbers (i.e., colony forming units; cfu) using the spread plate method to quantify rhizobia (Somasegaran and Hoben, 1994). That is, each time the OD was measured, serial dilutions of each strain were prepared and plated on YMA with the plates incubated at room temperature for four days. Plates (n = 3 for each dilution) yielding a cell count between 30 and 300 cfu per plate were counted (Somasegaran and Hoben, 1994) and calibration curves prepared. Using the calibration curves, a target population of 8.0×10^8 cfu mL⁻¹ was set at the beginning of the experiment.

Results

Congo Red Assay

None of the five *R. leguminosarum* strains took up the congo red dye and all strains appeared

translucent on the red medium (results not shown).

Gram-stain

The Gram-stain revealed that all five *R. leguminosarum* strains were Gram-negative. All colonies on the glass slides appeared red (results not shown). At 100× magnification the cells had a distinctive rod-shape consistent with *Rhizobium* (Somasegaran and Hoben, 1994).

After completing the congo red assay, the Gram stain, and observing cell morphology, it was concluded that the bacterial cultures were consistent with *Rhizobium*.

Growth curves

Growth curves were plotted for each *R. leguminosarum* strain using the OD readings and time after inoculation when readings were taken (Figure A.1). The spread plate cell counts were correlated with the readings to obtain OD values that represented actual bacterial cell counts for each *Rhizobium* strain. The spread plate cell counts were logarithmically transformed and graphed against the OD values to obtain a line equation for each *Rhizobium* strain, which could be used to calculate an OD value for the target cell concentration (Figure A.2). For subsequent experiments, the OD value and the growth curve line equation were used to estimate the rhizobial populations in real-time (Table A.1). Due to the nature of bacterial growth an OD range of ± 0.05 was allowed.

Discussion

All five isolates chosen for use in the main studies were found to be Gram-negative, rod-shaped bacteria that did not take up congo red dye; thus confirming their status as *R. leguminosarum*. The target population of 8.0×10^8 cfu mL⁻¹ occurred during the mid- to late-log growth phase for each *R. leguminosarum* strain.

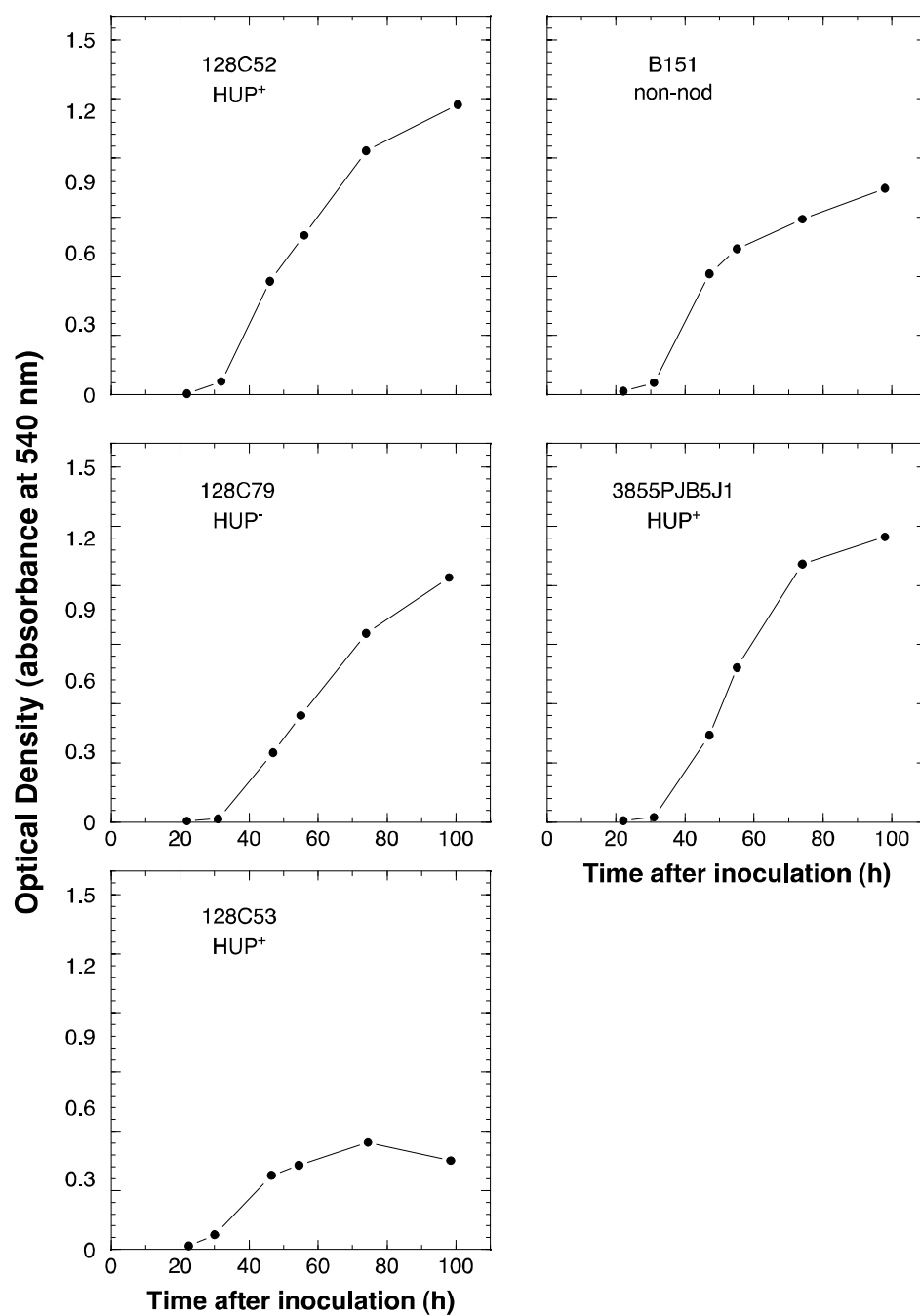


Figure A.1. Growth curves for each *R. leguminosarum* strain; optical density (absorbance at 540 nm) is plotted against time since inoculation (h).

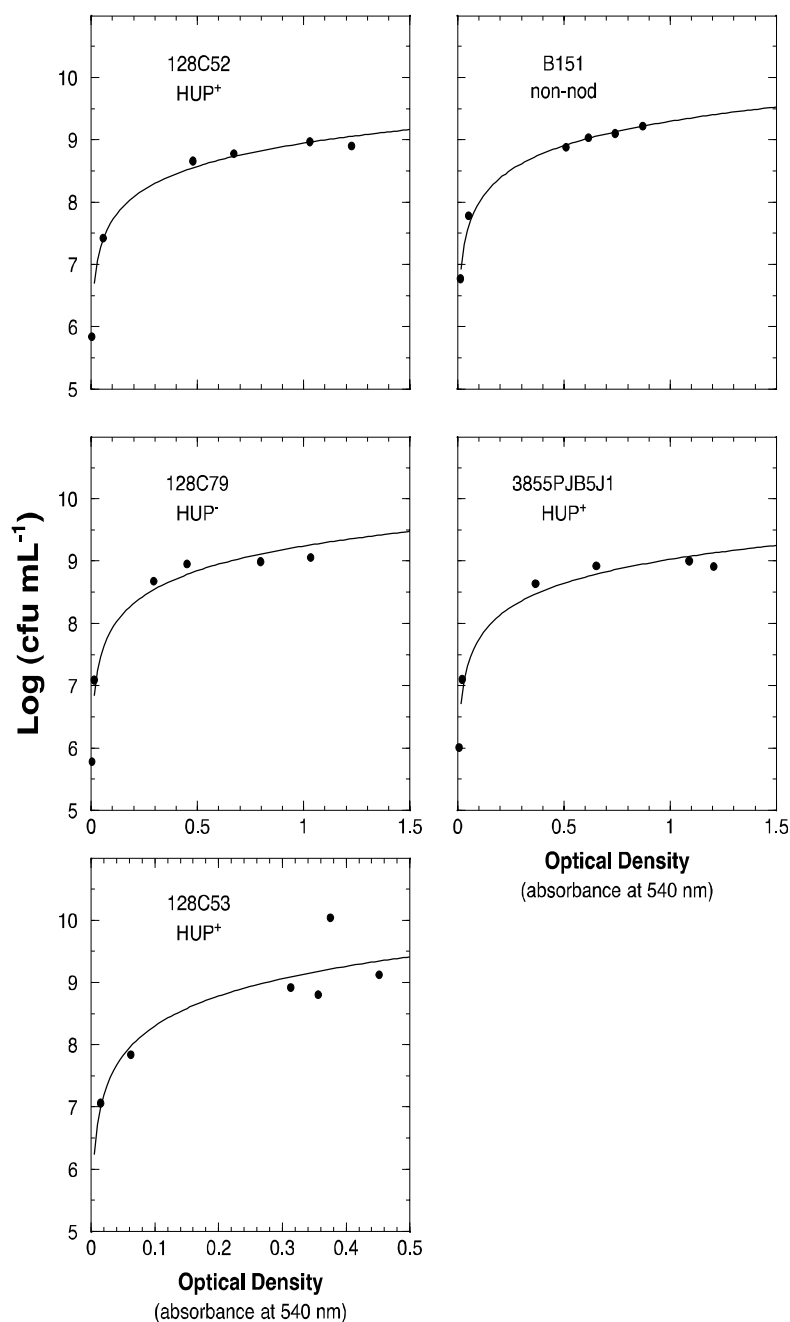


Figure A.2. Calibration curves for optical density (OD) absorbance values graphed against logarithmically transformed cell concentrations to illustrate growth of each *R. leguminosarum* strain. Line equations were determined for each *Rhizobium* strain and allowed cell concentrations to be calculated from OD values in subsequent experiments.

Table A.1. Target optical density (OD) value and cell concentration equations each *R. leguminosarum* strain.

<i>R. leguminosarum</i> strain	HUP status	Line equation [†]	R ²	Target OD range [‡]
128C52	+	$y = 8.95 + 1.25\log(x)$	0.995 ***	0.92 [§]
128C53	+	$y = 9.89 + 1.59\log(x)$	0.829 *	0.24
128C79	-	$y = 9.24 + 1.31\log(x)$	0.968 ***	0.55
3855PJB5J1	-	$y = 9.03 + 1.27\log(x)$	0.973 ***	0.79
B151	n/a	$y = 9.30 + 1.31\log(x)$	0.991 ***	0.50

*,*** indicate significance at the 0.05 and 0.001 level, respectively.

[†] a line equation, where x is OD, y is $\log(\text{cfu mL}^{-1})$.

[‡] OD (x) where $y = \log(8 \times 10^8 \text{ cfu mL}^{-1})$.

[§] acceptable OD values are ± 0.05 target OD value.

APPENDIX B: CONFIRMING HUP STATUS OF SELECT RHIZOBIAL STRAINS

Objective

The HUP status of each *Rhizobium* strain was confirmed prior to using it as a treatment in subsequent experiments. Rhizobial strains that are HUP⁺ are able to reduce methylene blue dye with the use of respiratory inhibitors (Haugland et al., 1983). Hydrogenase activity is not expressed in free-living *R. leguminosarum*, thus plant assays using nodules are used to assess the HUP status of rhizobia (Palacios et al., 1988). Palacios et al. (1988) showed that the methylene blue reduction assay can be used to successfully screen pea nodules by adapting methods by Haugland et al. (1983) and Lambert et al. (1985). Moreover, the methylene blue reduction assay is capable of screening a large number of nodules quickly (Lambert et al., 1985).

Materials and Methods

Plant tests

Pea (*Pisum sativum* cv. CDC Meadow) seeds were surface sterilized with ethanol and sodium hypochlorite in the same manner as described in Section 3.2.2 (Palacios et al., 1988; Somasegaran and Hoben, 1994), germinated on moist filter paper, inoculated with one of the *R. leguminosarum* strains and planted in Leonard jar assemblies (Section 3.2.2) (Somasegaran and Hoben, 1994). The Leonard jars contained sterilized silica sand supplemented with Fahraeus N-free growth medium (Somasegaran and Hoben, 1994; Vincent, 1970). One seedling was planted in each Leonard jar; each treatment was replicated four times. Plants were grown for four weeks in a growth chamber with a day/night temperature of 24°C/21°C and day/night length of 16 h/8 h. Roots were washed and fresh nodules removed from the roots (Lambert et al., 1985; Palacios et al., 1988).

Bacterial strains and growth media

Four treatments were applied to pea seeds: *R. leguminosarum* strains 128C52, 128C53, 128C79, and 3855PJB5J1. Sterile water and B151, used as control treatments, did not produce nodules on the pea roots and therefore could not be analyzed with the methylene blue reduction assay. The four *R. leguminosarum* cultures were grown in YMB and the cell concentration was determined using OD values as described in Appendix A. Pea seeds were inoculated with

rhizobial strains immediately before planting (Table B.1).

Table B. 1. Estimated *R. leguminosarum* cell concentration applied to pea seeds at time of planting for methylene blue reduction assay.

<i>R. leguminosarum</i>	Optical density at planting	Rhizobia population at planting cfu seed ⁻¹
128C52	0.7631	0.79×10^8
128C53	0.3118	1.52×10^8
128C79	0.4761	0.82×10^8
3855PJB5J1	0.7095	0.86×10^8

Methylene blue reduction assay

The methylene blue solution contains 200 mM iodoacetic acid, 200 mM malonic acid, 10 mM methylene blue, 50 mM KH₂PO₄, and 2.5 mM MgCl₂, adjusted to pH 5.6 with KOH (Haugland et al., 1983). Sterilized filter papers in Petri dishes were saturated with the methylene blue solution and nodules placed on the filter paper. A separate Petri dish was used for each rhizobial strain. The nodules were crushed into the filter paper with the end of a glass rod (Lambert et al., 1985) and the crushed nodules were allowed to sit under ambient conditions for 15 min before being placed in a Torbal anaerobic jar (Model AJ-2) that was then flushed three times with 5% H₂ in N₂ and left overnight to incubate. The assay indicates hydrogenase activity; nodules containing HUP⁺ rhizobia are able to reduce the blue dye and produce white areas around the crushed nodule (Lambert et al., 1985). The anaerobic jar was opened the following morning and the Petri dishes were inspected for color change and photographed.

Results

HUP status of rhizobial strains

The HUP⁺ strains, 128C52 and 128C53, had visible white discoloration where the nodules had been crushed, confirming that the two strains possessed the hydrogenase enzyme (Figure B.1). Conversely, strains 128C79 and 3855PJB5J1 produced no color reaction, indicating that these strains were HUP⁻ (Figure B.1). The HUP status of the *R. leguminosarum* strains was used as a treatment in subsequent experiments.

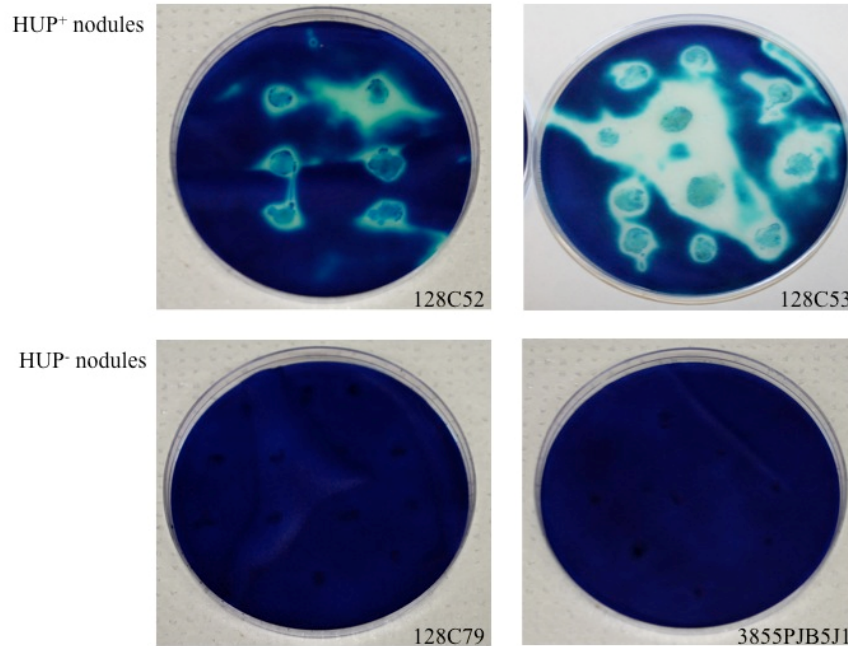


Figure B. 1. A photograph of the Petri dishes that were used in the methylene blue reduction assay. Nodules were removed from pea plants that had been grown for four weeks and inoculated with four nodulating *R. leguminosarum* strains. Decoloration around nodules indicates a rhizobia strain is HUP⁺; no decoloration indicates HUP⁻ rhizobia strains.

APPENDIX C: RHIZOSPHERE GAS SAMPLING PARAMETERS

Objective

A preliminary study was conducted to establish parameters for rhizosphere gas sampling in the greenhouse study. Two trials were conducted to determine: i) how long it took N_2O to diffuse into the platinum-cured silicone tubing and ii) whether replacing the air in the tubing while collecting a gas sample compromised the sample.

Materials and Methods

Diffusion test

The acrylic pots used for the greenhouse study were designed with two gas sampling ports in the side of the pot that were attached to a coil of gas permeable silicone tubing used to sample the atmosphere in the rhizosphere. The pots are described in detail in Section 3.2.3.1. The tubing used for rhizosphere sample collection was a gas permeable, water impermeable, platinum-cured silicone tubing (Tygon®). Although it was well known that N_2O can diffuse through the wall of the tubing, there was no information available as to how long it took for the air inside the tubing to reach equilibrium with the external air. Thus, an airtight chamber was fitted with one of the rhizosphere gas sampling coils and support structure, which was composed of four plastic rods attached to two metal rings with 150 cm of tubing wrapped around it (Figure C.1). The airtight chamber was used in order to determine the time required for the internal and external atmospheres to reach equilibrium. The chamber had an internal volume of 2.94 L; the internal volume of the silicone of tubing (150 cm \times 0.478 cm i.d.) was 26.9 mL.

The diffusion test began by flushing the chamber for 8 min with 100 ppmv N_2O and collecting a sample of the chamber atmosphere. The silicone tubing was flushed with approximately two volumes (i.e., 60 mL) of UHP N_2 . The tubing was allowed to equilibrate for 7-, 14-, or 21-min before a sample was collected from both the tubing and the surrounding chamber atmosphere. During sample collection, a 60 mL syringe filled with UHP N_2 was connected to one end of the silicone tubing via a gas sampling port installed in the wall of the acrylic chamber. A 20 mL syringe was connected to the other end of the tubing via a second gas sampling port and the air in the tubing withdrawn into the syringe. In this way, UHP N_2 from the 60-mL syringe was drawn into the tubing to replace the air being removed and to prevent the

tubing from collapsing. After sample collection, the entire chamber was opened to ambient air and reset by flushing the chamber for 8 min with 100 ppmv N₂O. The entire procedure was repeated a total of three times; however, the amount of time the tubing was allowed to equilibrate was extended each time from 7 min to 14 min and then to 21 min. Samples from the chamber and the tubing were collected for each time. All samples were analyzed for N₂O on a Varian CP-3800 Gas Chromatograph (GC) (Varian, Walnut Creek, CA).



Figure C. 1. Airtight chamber used to test N₂O diffusion from the chamber into the silicone tubing.

Sample collection test

A preliminary test was conducted to determine what impact the gas in the flushing syringe (i.e., UHP N₂) would have on the gas collected at the sampling syringe. In this test, the flushing syringe was filled with 60 mL of air containing 1 ppmv sulfur-hexa-fluoride (SF₆). Sulfur-hexa-fluoride was chosen as it is chemically and biologically inert, and is often used as a tracer in greenhouse gas studies (Verburg et al., 2004). In addition, the electron capture detector (ECD) used to detect and quantify N₂O is also highly sensitive to SF₆. The test was repeated three times and three separate samples were collected from the same chamber shown in Figure C.1.

Results

Diffusion test

Results of the diffusion test indicate that equilibrium between the atmospheres internal and external to the silicone tubing reached equilibrium in about 14 min (Table C.1).

Table C. 1. Nitrous oxide concentrations in chamber and tubing for three different time intervals to determine time required for N₂O to diffuse into tubing and equilibrate with the chamber.

Equilibration time	Chamber N ₂ O	Tubing N ₂ O
min	ppmv	
7	92.44	73.06
14	87.66	87.40
21	96.85	93.39

Sample collection test

Gas samples collected from the silicone tubing contained very small amounts of SF₆ (Table C.2), indicating that there was very little mixing of the gases in the tubing during sample collection. Indeed, the SF₆ concentrations in the gas samples were about 100× lower than the concentration in the reservoir (i.e., in the flushing syringe).

Table C.2. Sulfur-hexa-fluoride (SF₆) concentration in gas samples used to evaluate the effectiveness of rhizosphere gas sampling system.

Sample	SF ₆ concentration
	ppbv
SF ₆ test 1	n/a [†]
SF ₆ test 2	14.7
SF ₆ test 3	8.9

[†] Syringe used for sample collection became plugged during collection, which compromised the sample.

Conclusion

The silicone tubing used for gas sampling in the rhizosphere reached an equilibrium with its surrounding atmosphere within 14 min after being flushed with UHP N₂. In soil, however, the diffusion of air to the chamber may be slowed due to a variety of physical factors (e.g., bulk density, porosity, tortuosity, and water content); therefore, the time interval between flushing the

tubing and collecting a rhizosphere gas sample was extended to 30 min to ensure that there had been adequate time for equilibration. A longer equilibration time also was necessary to allow for the flushing and sampling of multiple pots. The SF₆ test demonstrated that using UHP N₂ to replace the volume drawn off from the rhizosphere tubing did not compromise or adversely impact the sample.

APPENDIX D: HYDROGEN EXPOSURE RATE AND UPSCALING H₂ CALCULATIONS

Equation variables

HE _x R	hydrogen exposure rate	μmol H ₂ cm ⁻³ h ⁻¹
NF	nitrogen fixation	μmol N ₂ ha ⁻¹ season ⁻¹
EAC	electron allocation coefficient	
SL	season length	h season ⁻¹
D	nodule depth	cm
L	total row length	cm ha ⁻¹
W	total width affected by nodules	cm

Equation

$$\text{HE}_{\text{x}}\text{R} = [(3\text{NF} \times (1 - \text{EAC})) / (\text{EAC} \times \text{SL})] / (\text{D} \times \text{L} \times \text{W})$$

Table D. 1. Hydrogen exposure rate (HExR) calculations for field pea grown in Saskatchewan. Three N fixation rates and three distances from nodule (W) were used to determine a range of HExRs for pea.

N fixed by pea crop	NF	EA C	Growing season	N fixing hours	SL	D	Rows	L	W	HExR	HExR
kg N ha ⁻¹ season ⁻¹	μmol N ₂ ha ⁻¹ season ⁻¹		d	h d ⁻¹	h season ⁻¹	cm	rows ha ⁻¹	m ha ⁻¹	c m	μmol H ₂ cm ⁻³ h ⁻¹	nmol H ₂ cm ⁻³ h ⁻¹
75	2.68×10^9	0.67	56	16	896	10	444	44400	2	4.97×10^{-2}	49.7
75	2.68×10^9	0.67	56	16	896	10	444	44400	4	2.49×10^{-2}	24.9
75	2.68×10^9	0.67	56	16	896	10	444	44400	8	1.24×10^{-2}	12.4
123	4.39×10^9	0.67	56	16	896	10	444	44400	2	8.15×10^{-2}	81.5
123	4.39×10^9	0.67	56	16	896	10	444	44400	4	4.08×10^{-2}	40.8
123	4.39×10^9	0.67	56	16	896	10	444	44400	8	2.04×10^{-2}	20.4
179	6.39×10^9	0.67	56	16	896	10	444	44400	2	1.19×10^{-1}	118.7
179	6.39×10^9	0.67	56	16	896	10	444	44400	4	5.93×10^{-2}	59.3
179	6.39×10^9	0.67	56	16	896	10	444	44400	8	2.97×10^{-2}	29.7

Table D. 2. Upscaled H₂ production rates for field pea over a growing season. Values were calculated for three pea N fixation values.

W	N fixed by pea crop	Hydrogen Exposure Rate						
		nmol H ₂ cm ⁻³ h ⁻¹	nmol H ₂ cm ⁻³ day ⁻¹	nmol H ₂ day ⁻¹ ha ⁻¹	mol H ₂ day ⁻¹ ha ⁻¹	m ³ H ₂ day ⁻¹ ha ⁻¹	L H ₂ day ⁻¹ ha ⁻¹	L H ₂ ha ⁻¹ season ⁻¹
cm	kg N ha ⁻¹ season ⁻¹							
2	75	49.7	795.5	7.06×10^{10}	70.6	1.60	1604	89845
4	75	24.9	397.8	7.06×10^{10}	70.6	1.60	1604	89845
8	75	12.4	198.9	7.06×10^{10}	70.6	1.60	1604	89845
2	123	81.5	1304.7	1.16×10^{11}	115.9	2.63	2631	147346
4	123	40.8	652.3	1.16×10^{11}	115.9	2.63	2631	147346
8	123	20.4	326.2	1.16×10^{11}	115.9	2.63	2631	147346
2	179	118.7	1898.7	1.69×10^{11}	168.6	3.83	3829	214430
4	179	59.3	949.3	1.69×10^{11}	168.6	3.83	3829	214430
8	179	29.7	474.7	1.69×10^{11}	168.6	3.83	3829	214430